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THÈSE

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PRINCIPALES ABREVIATIONS

ACoA-LC	Acyl-coenzyme A à longue chaîne
Acyl-CoA	Acyl-coenzyme A
AG	Acide gras
Akt/PKB	Protéine kinase B
AMPK	Protéine kinase activée par l'AMP
ASC	*Adipose stromal cell*
ATGL	*Adipose triglyceride lipase*
ATP	Adénosine triphosphate
CD15	Cluster de différenciation 15
CD36	Cluster de différenciation 36
CD56	Cluster de différenciation 56
CER	Céramide
CGI-58	*Comparative gene identification-58*
DAG	Diacylglycérol
DGAT	*DAG acyltransferase*
DT2	Diabète de type 2
EPS	*Electrical pulse stimulation*
ERK	*Extracellular signal-regulated kinases*
FAP	Progéniteur fibro/adipogénique
FATP1	*Fatty acid transport protein 1*
G0S2	G_0/G_1 Switch Gene 2
GLUT4	*Glucose transporter 4*

GPAT	*Gycerol-3-phosphate acyltransferase*
IL-4	Interleukine-4
IL-6	Interleukine-6
IMAT	Tissu adipeux intramusculaire
IMC	Indice de masse corporelle
IMTG	Triacylglycérol intramyocellulaire
IRS-1	*Insulin receptor substrate-1*
LHS	Lipase hormone-sensible
MAG	Monoacylglycérol
MGL	Monoacylglycérol lipase
NO	Monoxide d'azote
PDK4	*Pyruvate dehydrogenase kinase 4*
PGC1α	*Peroxysome proliferator-activated receptor γ coactivator 1 α*
PI3K	*Phosphatidylinositol 3-kinase*
PKA	Protéine kinase A
PKC	Protéine kinase C
PKR	*Double-stranded RNA-activated protein kinase*
PLIN5	Périlipine 5
PP2A	Protéine phosphatase 2A
PPARβ	*Peroxysome proliferator-activated receptor β*
SVF	Fraction stroma-vasculaire
TAG	Triacylglycérol
TNF-α	*Tumor necrosis factor-α*

1. CONTEXTE

L'obésité constitue un problème majeur de santé publique, et est en constante augmentation dans le monde. Les dernières estimations indiquent qu'en 2014 près de 2 milliards d'individus sur la planète étaient en surpoids, dont 600 millions d'obèses. L'obésité, définie comme une accumulation anormale ou excessive de graisse corporelle qui représente un risque pour la santé, a été reconnue comme une maladie par l'Organisation Mondiale de la Santé (OMS) en 1997. De façon marquante, le surpoids et l'obésité sont de nos jours responsables de davantage de décès que ne l'est l'insuffisance pondérale.

L'obésité est un facteur de risque prédisposant au développement de nombreuses pathologies chroniques telles que des maladies cardiovasculaires et métaboliques, mais également certains cancers (Pi-Sunyer, 2009). Une des principales pathologies liées à l'obésité est le diabète de type 2. Cette pathologie est caractérisée par une dérégulation de l'homéostasie glucidique (hyperglycémie à jeun), due à une perte progressive de la sensibilité des tissus à l'insuline (*i.e.* insulino-résistance), notamment au niveau du foie, du tissu adipeux et du muscle squelettique (DeFronzo *et al.*, 1982; Defronzo *et al.*, 1981).

Plusieurs hypothèses, non mutuellement exclusives, ont été proposées pour expliquer le lien entre obésité et insulino-résistance, parmi lesquelles le développement d'une inflammation locale ou bien, et ce qui a fait l'objet de mon travail de thèse, une insulino-résistance induite l'accumulation excessive de lipides ou « lipotoxicité » (Samuel and Shulman, 2012). En temps normal, les lipides alimentaires sont préférentiellement stockés sous forme de triacylglycérols (TAG) au sein du tissu adipeux. Cependant, lorsque l'obésité se développe, le tissu adipeux croît jusqu'à une certaine limite, à partir de laquelle il n'est plus à même de tamponner cet excès de lipides. En effet, il semble que le tissu adipeux puisse se développer jusqu'à une certaine limite, propre à chaque individu ; il s'agit de la théorie de l'expandabilité du tissu adipeux (Virtue and Vidal-Puig, 2010). Les lipides

en excès ne pouvant plus y être stockés vont alors se déposer dans d'autres organes, non destinés au stockage (pancréas, cœur, foie, muscle squelettique…) : c'est ce que l'on appelle le stockage ectopique de lipides. Ce dernier a été associé au développement d'une lipotoxicité et d'une altération de la sensibilité à l'insuline dans de nombreux organes impliqués dans la régulation de l'homéostasie glucidique (Lettner and Roden, 2008).

En situation physiologique, après un repas, la glycémie augmente et l'insuline sécrétée par le pancréas va agir sur ses tissus cibles afin de ramener la glycémie à une valeur normale (autour de 1 g/L). Ainsi, l'insuline va d'une part inhiber la production hépatique de glucose, et d'autre part stimuler le transport de glucose dans le tissu adipeux et le muscle squelettique (figure 1). Il a été démontré qu'en situation postprandiale, le muscle est responsable de 80% du transport de glucose en réponse à l'insuline, lui conférant un rôle central dans la gestion de l'homéostasie glucidique (DeFronzo and Tripathy, 2009; Ferrannini *et al.*, 1988). Ainsi, une perte de sensibilité musculaire à l'insuline entraîne une insulino-résistance systémique conduisant au diabète de type 2. Comprendre les mécanismes à l'origine du développement de l'insulino-résistance musculaire chez des individus obèses apparaît donc essentiel dans la prévention/le traitement du diabète de type 2.

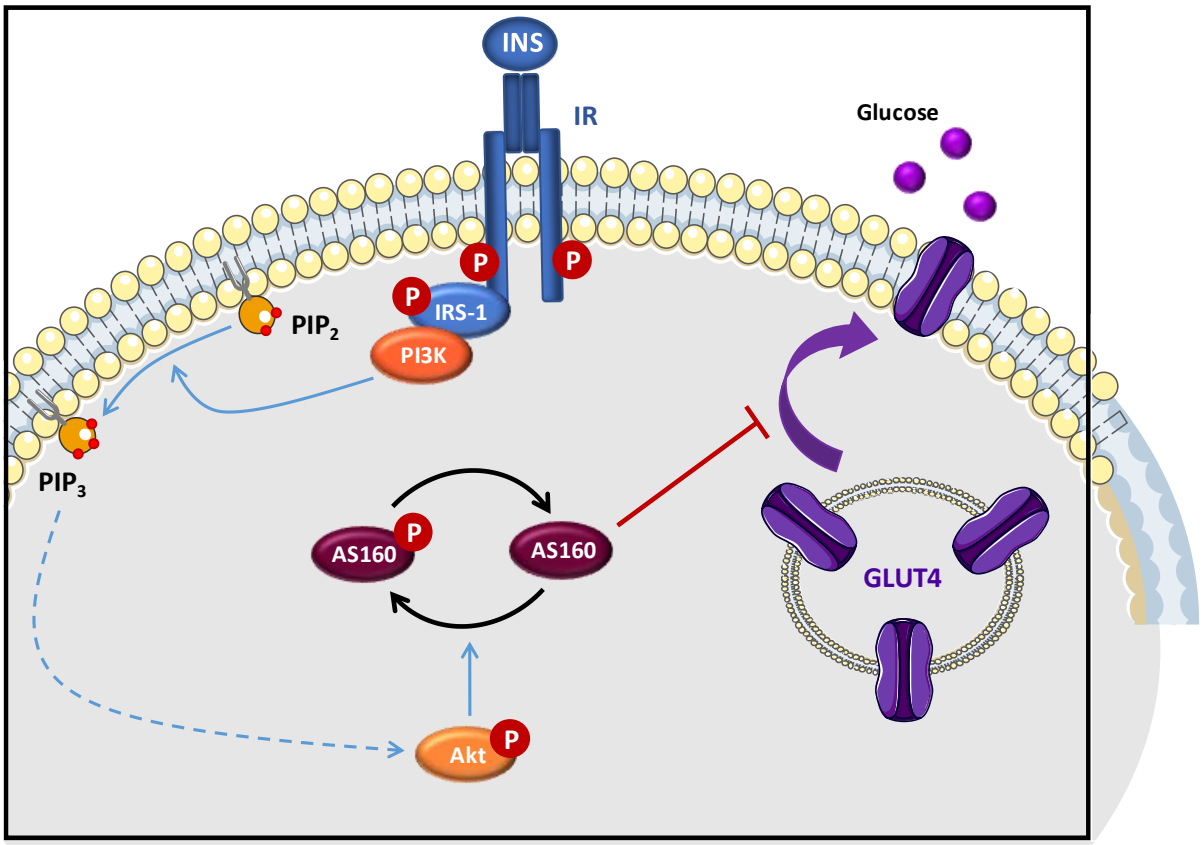

Figure 1. Voie de signalisation de l'insuline

La fixation de l'insuline (INS) induit l'auto-phosphorylation de son récepteur (IR) sur des résidus tyrosine (tyr). Ceci conduit au recrutement d'*insulin receptor substrate 1* (IRS-1), qui va alors être activé par phosphorylation sur un résidu tyrosine 612. Ce dernier va activer la phosphatidylinositol 3-kinase (PI3K), conduisant à la phosphorylation du phosphatidylinositol diphosphate (PIP_2) en phosphatidylinositol triphosphate (PIP_3). Ce dernier va permettre l'activation d'Akt par phosphorylation sur des résidus sérine 403 et thréonine 308. Akt va alors phosphoryler AS160 (*Akt substrate of 160 kDa*), permettant ainsi aux vésicules de *glucose transporter 4* (GLUT4) de transloquer à la membrane plasmique. (Saltiel and Pessin, 2002)

De nombreuses études ont mis en évidence un lien entre accumulation ectopique de lipides dans le muscle, insulino-résistance et diabète de type 2. Il a été montré que les lipides peuvent être présents sous deux formes dans le muscle squelettique (figure 2) :

- soit sous forme d'adipocytes insérés entre les fibres/faisceaux musculaires, appelé tissu adipeux intramusculaire (IMAT)

- soit sous forme de gouttelettes lipidiques à l'intérieur des fibres musculaires, il s'agit des triacylglycérols intramyocellulaires (IMTG)

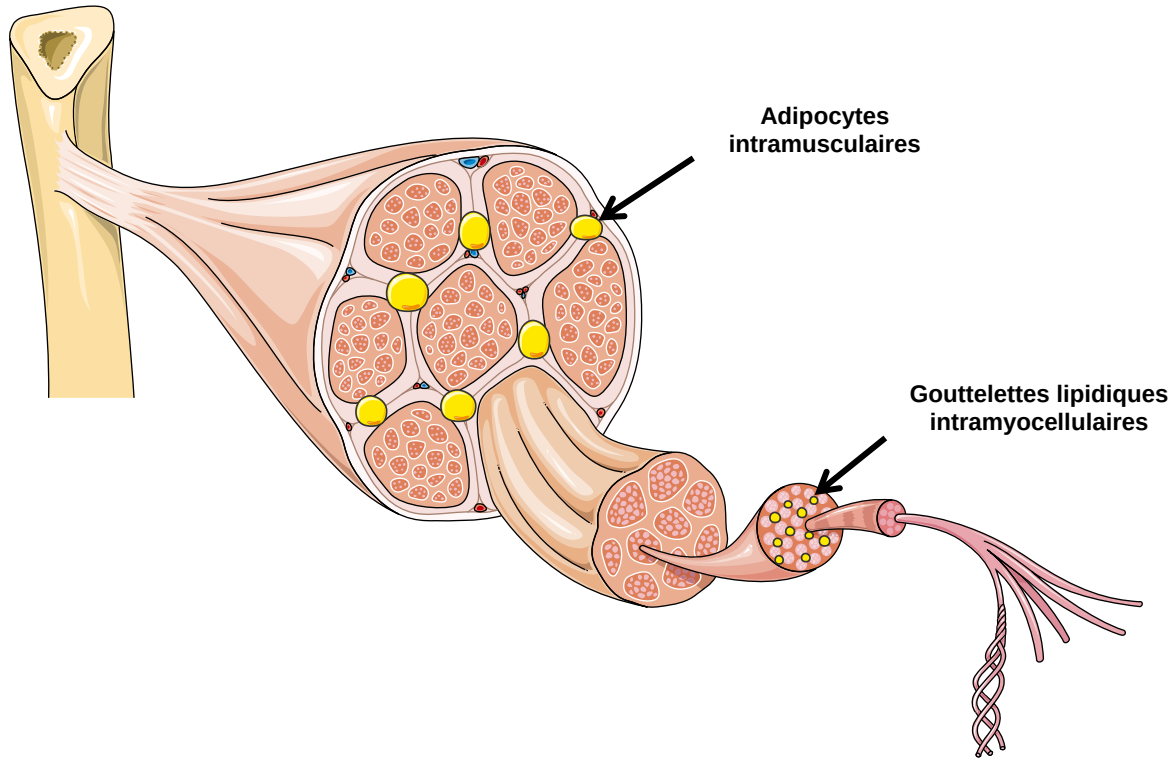

Figure 2. Schéma représentant les différentes formes de dépôts lipidiques musculaires
Dans le muscle squelettique, les lipides sont stockés soit sous forme de cellules adipeuses localisées entre les faisceaux de fibres musculaires (*i.e.* adipocytes intramusculaires), soit sous forme de gouttelettes lipidiques à l'intérieur des cellules musculaires (*i.e.* gouttelettes lipidiques intramyocellulaires).

Une des thématiques de l'équipe est de comprendre comment se développe l'insulino-résistance musculaire associée à l'obésité, et notamment le lien entre l'accumulation de lipides musculaires et l'altération de la sensibilité à l'insuline. Ainsi, le but de ce travail de thèse a été d'étudier les mécanismes liant accumulation d'IMAT et d'IMTG et insulino-résistance au sein du muscle squelettique.

2. PHYSIOLOGIE DU MUSCLE SQUELETTIQUE

Le système musculaire est composé de trois types de muscles :

- Le muscle cardiaque, dont le rôle est d'assurer la propulsion du sang dans tout l'organisme *via* le système circulatoire. Sa contraction est contrôlée de façon involontaire, et régulée de façon permanente par le système nerveux autonome et certaines hormones (*i.e.* adrénaline, noradrénaline, hormones thyroïdiennes).
- Les muscles lisses, présents au sein des vaisseaux et de certains organes creux, assurent de nombreuses fonctions au sein de l'organisme (*i.e.* régulation du flux sanguin *via* la vasoconstriction et la vasodilatation des vaisseaux, contrôle du tractus gastro-intestinal et du tractus respiratoire). Ils se contractent de façon involontaire sous le contrôle du système nerveux autonome.
- Les muscles squelettiques, recouvrant l'ensemble du squelette, permettent le maintien postural et la réalisation de mouvements. Ils présentent une structure striée caractéristique, et leur contraction est contrôlée de façon volontaire par le système nerveux somatique. Le corps humain contient plus de 600 muscles squelettiques, représentant environ 40% du poids corporel chez l'homme et environ 35% chez la femme.

2.1. STRUCTURE DU MUSCLE SQUELETTIQUE

Les muscles squelettiques, attachés aux os par des tendons, sont composés de tissu musculaire (*i.e.* fibres musculaires) permettant la contraction, de tissu conjonctif (*i.e.* endomysium, perimysium et epimysium) ,assurant le maintien de la structure du muscle et la transmission du mouvement aux pièces osseuses, de tissu nerveux (*i.e.* motoneurones) contrôlant la contraction et le tonus musculaire et de vaisseaux sanguins permettant l'apport d'oxygène et de nutriments ainsi que l'élimination des déchets (Astrand and Rodahl, 1973; Grassé *et al.*, 1968).

Chaque muscle est recouvert de l'épimysium et est composé de nombreux faisceaux de fibres, chacun étant délimité par le périmysium. Chaque faisceau

contient de nombreuses fibres musculaires, entre lesquelles est présent une matrice extracellulaire appelée endomysium (figure 3).

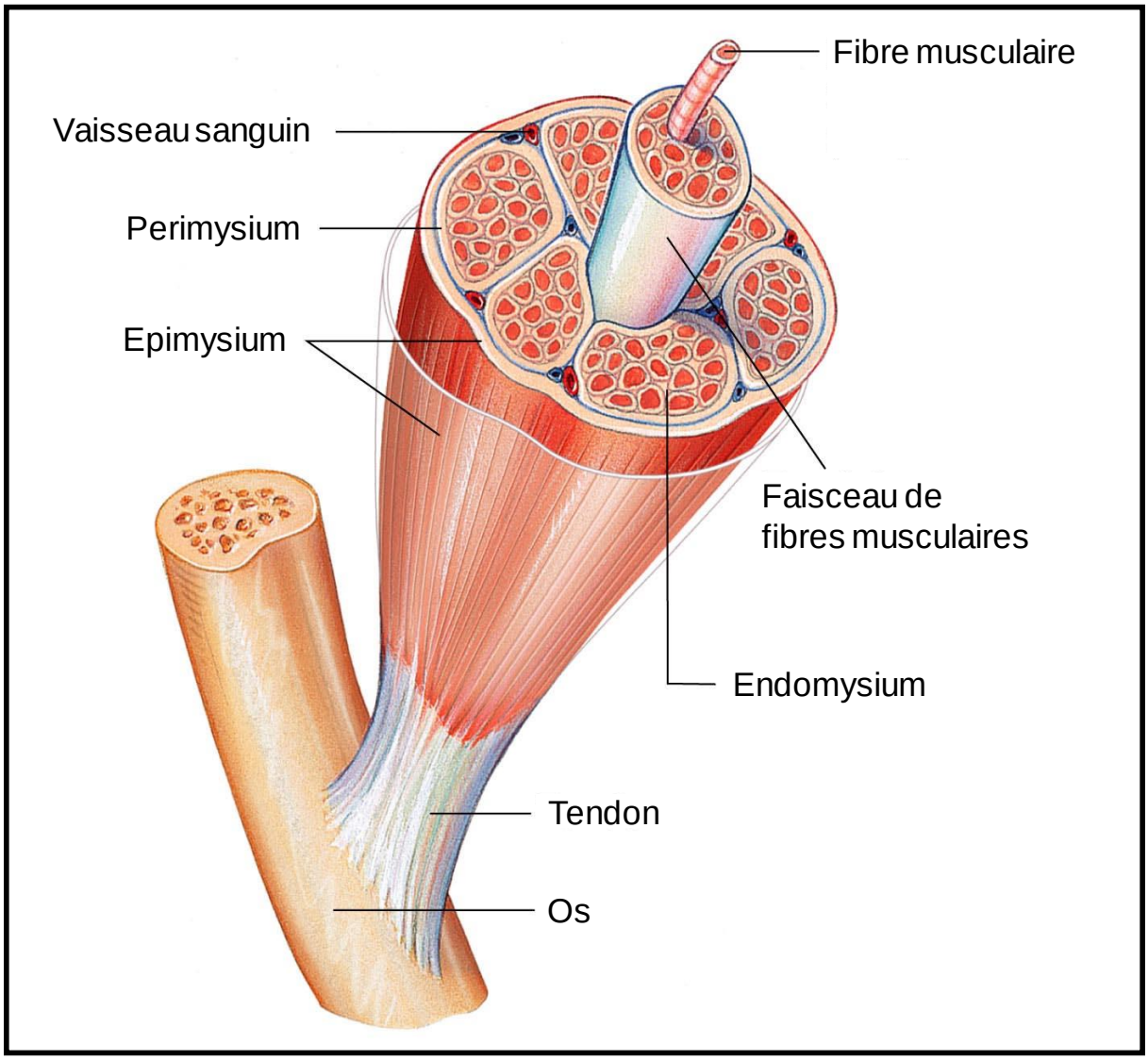

Figure 3. Structure du muscle squelettique

Les muscles squelettiques (à l'exception des muscles orbiculaires des lèvres et des muscles peauciers) se rattachent aux os par des tendons. Ils sont entourés d'une gaine de tissu conjonctif appelée épimysium, et irrigués par la présence de nombreux vaisseaux sanguins. Un muscle se décompose en plusieurs faisceaux de fibres, délimités par le périmysium. Chaque faisceau contient de nombreuses fibres musculaires autour desquelles est présent un tissu conjonctif appelé endomysium.

Chaque fibre musculaire est entourée d'une membrane appelée sarcolemme, constituée d'une lame basale et de la membrane plasmique (Kühnel, 2003). Les cellules souches musculaires, appelées cellules satellites, sont insérées entre la lame basale et la membrane plasmique et permettent la régénération musculaire suite à une lésion (Muir *et al.*, 1965). La différenciation musculaire implique la fusion

de nombreux progéniteurs myocytaires appelés myoblastes (*i.e.* cellules satellites activées) conduisant à la formation de fibres matures multinuclées. Les noyaux de ces cellules sont répartis de façon hélicoidale sous le sarcolemme. Le cytoplasme des fibres musculaires, appelé sarcoplasme, contient notamment des mitochondries, des réserves énergétiques (sous forme de gouttelettes lipididiques et de glycogène) et de nombreuses myofibrilles entourées de réticulum sarcoplasmique (figure 4).

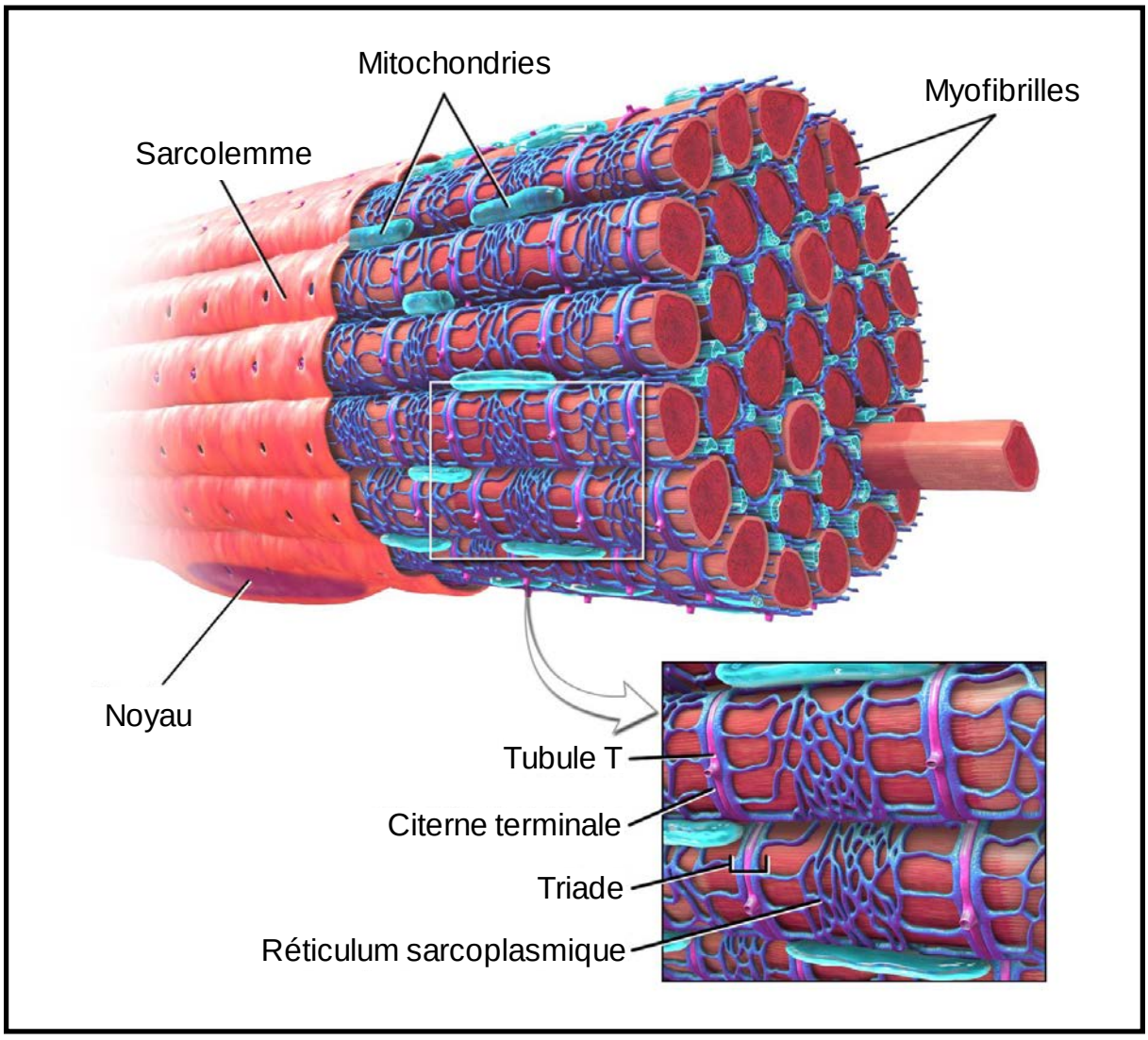

Figure 4. Structure d'une fibre musculaire

Chaque fibre est délimitée par une membrane appelée sarcolemme, constituée d'une lame basale et d'une membrane plasmique, sous laquelle sont insérés les noyaux. De nombreuses myofibrilles, entourées de réticulum sarcoplasmique et entre lesquelles sont insérées de nombreuses mitochondries, sont présentes dans chaque fibre musculaire. Des invaginations du sarcolemme, appelées tubules T, permettent la conduction des impulsions électriques à l'ensemble des myofibrilles. Chaque tubule T est entouré de deux portions élargies du réticulum sarcoplasmique, appelées citernes terminales. L'ensemble constitué par un tubule T et deux citernes terminales est appelé triade.

Chaque myofibrille est entourée d'invaginations transversales du sarcolemme appelées tubules T et de réticulum sarcoplasmique, qui constitue une réserve de calcium. Le réticulum sarcoplasmique est constitué de tubules disposés de façon longitudinale le long des myofibrilles, aux extrémités desquelles sont situées des citernes terminales. L'ensemble d'un tubule T et de deux citernes terminales est appelé triade et joue un rôle important dans le couplage excitation-contraction (Figure 4). Chaque fibre musculaire est innervée par un motoneurone. La synapse entre l'axone terminal d'un motoneurone et une fibre musculaire est appelée jonction neuro-musculaire. Lorsqu'une impulsion nerveuse arrive au niveau du bouton terminal de l'axone synaptique, un neurotransmetteur (l'acétylcholine) est sécrété et se fixe sur ses récepteurs présents au niveau du sarcolemme permettant l'ouverture de canaux ioniques et l'entrée de sodium dans la cellule. Ceci va entraîner une dépolarisation à l'origine de l'apparition d'un potentiel d'action qui va se propager le long du sarcolemme. Lorsque cette impulsion électrique arrive au niveau des tubules T, le potentiel d'action va permettre l'ouverture de canaux calciques voltage-dépendants de type L entraînant à leur tour l'ouverture de canaux calciques localisés aux niveau des citernes terminales du réticulum sarcoplasmique (*i.e.* récepteurs à la ryanodine) et entraîner la libération de calcium dans le sarcoplasme, permettant ainsi la contraction musculaire (Sandow, 1965).

Les myofibrilles sont composées de nombreuses unités fonctionnelles appelées sarcomères, qui sont délimités entre eux par des stries Z. Un sarcomère est composé de filaments épais de myosine et de filaments fins d'actine-troponine-tropomyosine (figure 5). La libération de calcium dans le sarcoplasme va permettre sa fixation sur la troponine, entraînant le basculement des molécules de tropomyosine et la libération des sites de liaison actine-myosine. Les têtes de myosine se lient à l'actine, basculent et tirent le filament fin d'actine vers l'intérieur du sarcomère avant de se détacher. La répétition de ce processus entraîne le glissement des filaments d'actine et de myosine l'un sur l'autre et le raccourcissement du sarcomère, caractéristique de la contraction musculaire. Il est important de noter que la tête de myosine possède un site de liaison pour l'ATP, qui fournit l'énergie nécessaire à la contraction. En effet, l'énergie issue de l'hydrolyse d'ATP en ADP est nécessaire pour lier la tête de myosine au filament d'actine et entraîner le glissement de ces derniers (Gordon *et al.*, 2000).

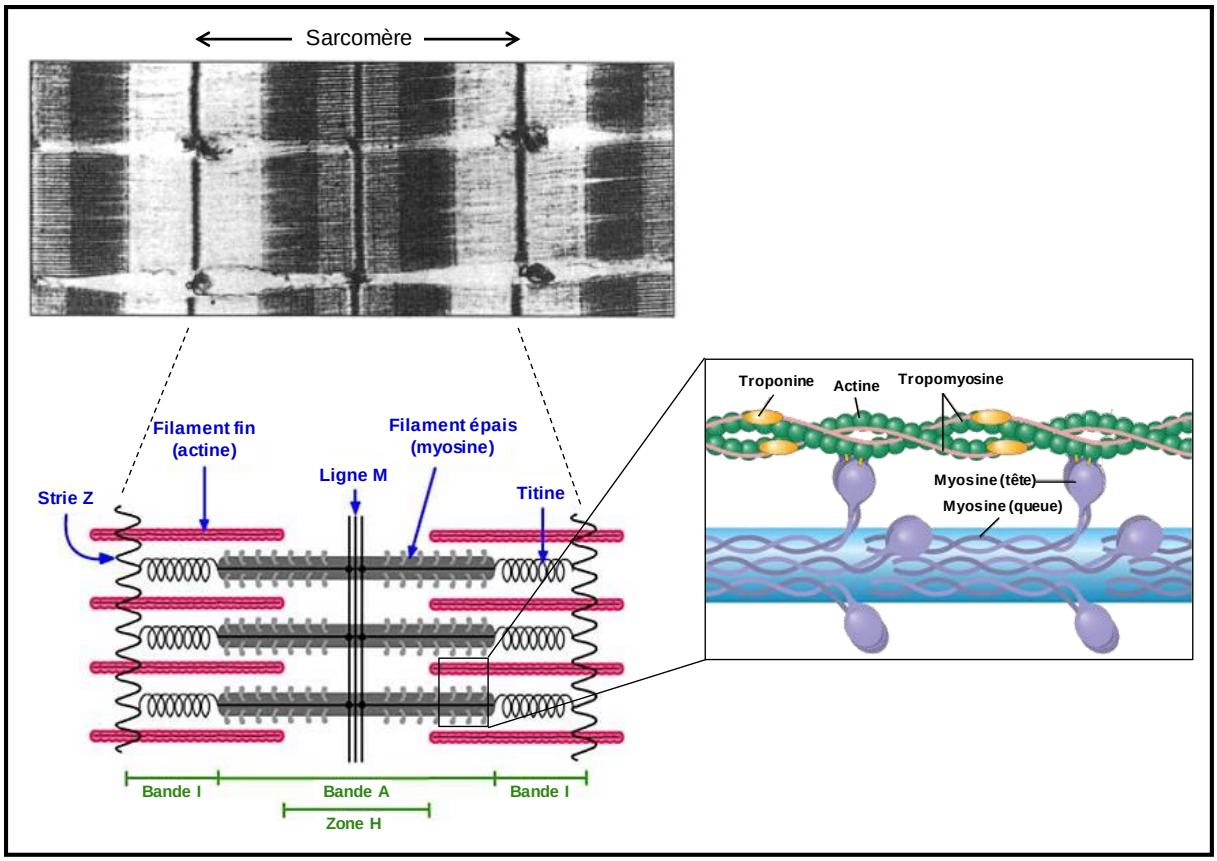

Figure 5. Structure d'un sarcomère

Les sarcomères constituent l'unité de base des myofibrilles, et leur organisation régulière est responsables de l'apparence striée des muscles squelettiques. Un sarcomère est le segment compris entre deux lignes foncées appelées stries Z (*i.e.* de l'allemand *zwischen* : « entre »). La bande I (*i.e.* isotropique) est accolée aux stries Z, suivie de la bande A (*i.e.* anisotropique), nommées d'après leurs propriétés physiques sous microscope polarisant. Une région plus claire est présente au centre de cette dernière, appelée zone H (*i.e.* de l'allemand *heller* : « plus pâle »), au centre de laquelle se situe une ligne plus foncée appelée ligne M (*i.e.* de l'allemand *mittel* : « centre »). Les myofibrilles sont composés principalement de trois types de filaments : les filaments épais de myosine, les filaments fins d'actine-troponine-tropomyosine et les filaments élastiques de titine reliant les filaments épais aux stries Z.

Enfin, l'arrêt de la contraction musculaire est contrôlé par le transport actif du calcium contenu dans le sarcoplasme à l'intérieur du réticulum sarcoplasmique par les pompes SERCA (*sarco/endoplasmic reticulum* Ca^{2+}*-ATPase*). La libération du calcium de la troponine va permettre à la tropomyosine de masquer à nouveau les sites de liaison de l'actine, interrompant l'accrochage des têtes de myosine à ces derniers, entraînant la relaxation de la fibre musculaire (Berchtold *et al.*, 2000).

2.2. METABOLISME ENERGETIQUE

Comme décrit ci-dessus, le bon fonctionnement du muscle squelettique nécessite un apport d'énergie sous forme d'ATP. Les deux principaux substrats énergétiques du muscle sont le glucose et les acides gras (AG) (figure 6).

Le glucose sanguin (*i.e.* d'origine alimentaire ou provenant de la dégradation du glycogène hépatique) est capté par les muscles squelettiques par le transporteur GLUT4 suite à l'augmentation postprandiale de la glycémie (en réponse à une stimulation des cellules musculaires par l'insuline) ainsi que de façon basale par le transporteur GLUT1 (Klip and Marette, 1992). Le glucose y est stocké sous forme de glycogène (*i.e.* glycogénogenèse), qui pourra être mobilisé (*i.e.* glycolyse) si les besoins en énergie augmentent, par exemple lors de la pratique d'un exercice physique. La glycolyse dégrade le glucose en pyruvate. Ce dernier pourra alors soit être transformé en lactate (*i.e.* glycolyse anaérobie lactique), soit entrer dans le cycle de Krebs et fournir de l'ATP via les phosphorylations oxydatives au niveau de la chaîne respiratoire des mitochondries (*i.e.* glycolyse aérobie). Cependant, les réserves de glycogène sont limitées et peuvent être rapidement épuisées lors d'une augmentation importante des besoins énergétiques. Les AG, d'origine circulante (*i.e.* provenant de la dégradation des réserves de triacylglycérols du tissu adipeux) ou stockés sous forme de triacylglycérols dans des gouttelettes lipidiques au sein des cellules musculaires (*i.e.* triacylglycérols intramyocellulaires, IMTG), constituent une autre source d'énergie. L'hydrolyse de ces gouttelettes (*i.e.* lipolyse) permet de fournir au muscle de l'énergie lors de la pratique d'un exercice physique d'intensité modérée. La production d'énergie à partir de substrats lipidiques nécessite l'apport d'oxygène pour synthétiser de l'ATP (*i.e.* aérobie). Les processus de synthèse et de dégradation des lipides musculaires seront décrits de façon détaillée dans la suite de ce manuscrit. Par ailleurs, de l'ATP peut être produit par l'hydrolyse des réserves de phosphocréatine en créatine par la créatine phosphokinase (*i.e.* anaérobie alactique). Ce processus a l'avantage de fournir de l'énergie de façon très rapide, mais les réserves de phosphocréatine sont extrêmement limitées (Wilmore and Costill, 2002).

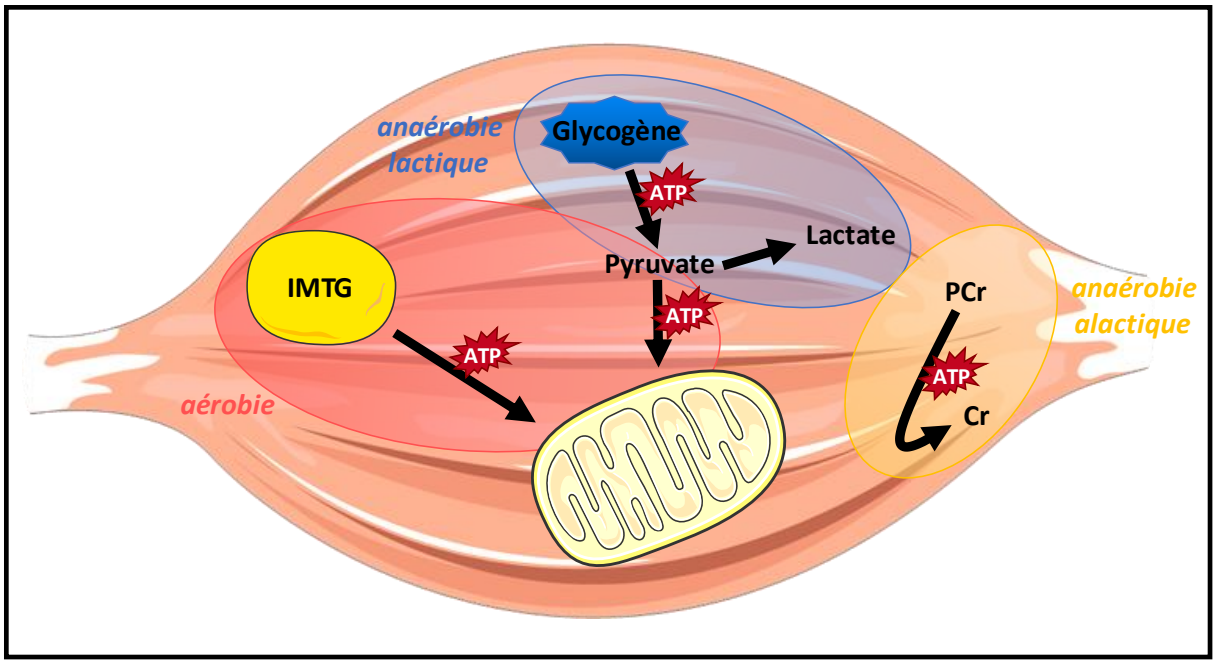

Figure 6. Principales voies de production d'énergie au sein du muscle squelettique

Le muscle squelettique peut produire de l'énergie chimique sous forme d'adénosine triphosphate (ATP) de différentes façons. En l'absence d'oxygène, de l'ATP peut être produit soit par l'hydrolyse de la phosphocréatine (PCr) en créatine (Cr) de façon anaérobie alactique (*i.e.* n'entraînant pas la production de lactate), soit par la dégradation des réserves de glycogène en pyruvate (*i.e.* glycolyse) qui sera alors transformé en lactate et éliminé, il s'agit de l'anaérobie lactique. En présence d'oxygène, l'ATP peut être produit par l'oxydation du pyruvate au sein des mitochondries ainsi que par la dégradation et l'oxydation des réserves de triacylglycérols intramyocellulaires (IMTG), il s'agit de processus aérobie.

Enfin, il est possible de produire de l'énergie à partir des protéines, les acides aminés contenus dans ces dernières pouvant en effet être transformés en acétyl coenzyme A, entrer dans le cycle de Krebs et être oxydés au niveau de la chaîne respiratoire mitochondriale. Il a été estimé que l'oxydation des protéines constitue environ 15% de la dépense énergétique de repos, et ne représente plus que 2 à 4 % de la dépense énergétique lors de la pratique d'un exercice physique (Lemon and Nagle, 1981). Cependant, bien que le pourcentage de protéines oxydées à l'exercice soit faible, la quantité totale (*i.e.* mesurée en grammes) est, considérant la forte augmentation de la dépense énergétique, supérieure à celle mesurée au repos et ne doit donc pas être négligée (figure 7). Prenons l'exemple d'un sujet (70kg) ayant au repos une dépense énergétique de 70 x 3.5 ml/min/kg = 0.245 l/min (soit 1.18 kcal/min ou 71 kcal/h) dont 15% est assurée par les protéines (10.65 kcal/h). Lorsque ce sujet (VO_2max de 4 l/min) effectue un exercice d'une heure à 75% de sa VO_2max

(3 l/min), sa dépense énergétique représente 869 kcal dont 4% est assurée par les protéines (34.76 kcal). Nous pouvons voir ainsi que durant l'exercice, l'oxydation des protéines a été multipliée par 3.3 par rapport au repos (34.76 kcal / 10.65 kcal). Nous constatons que sa dépense énergétique à l'exercice a été multipliée par 12 alors que l'utilisation des protéines en pourcentage a été divisée par 3.75. Cependant, la quantité de protéines oxydées a été multipliée par 4 par rapport à l'état de repos.

Protéines oxydées (grammes) = dépense énergétique x % protéines utilisées

X 3.3 X 12 ÷ 3.75

De façon intéressante, il a été mis en évidence que la pratique d'un exercice physique chez un sujet dont les réserves de glycogène musculaire ont été déplétées entraîne une forte augmentation de la quantité de protéines oxydées, ce qui confirme leur fonction de substrat énergétique, notamment lors de la pratique d'exercices de longue durée (Lemon and Mullin, 1980).

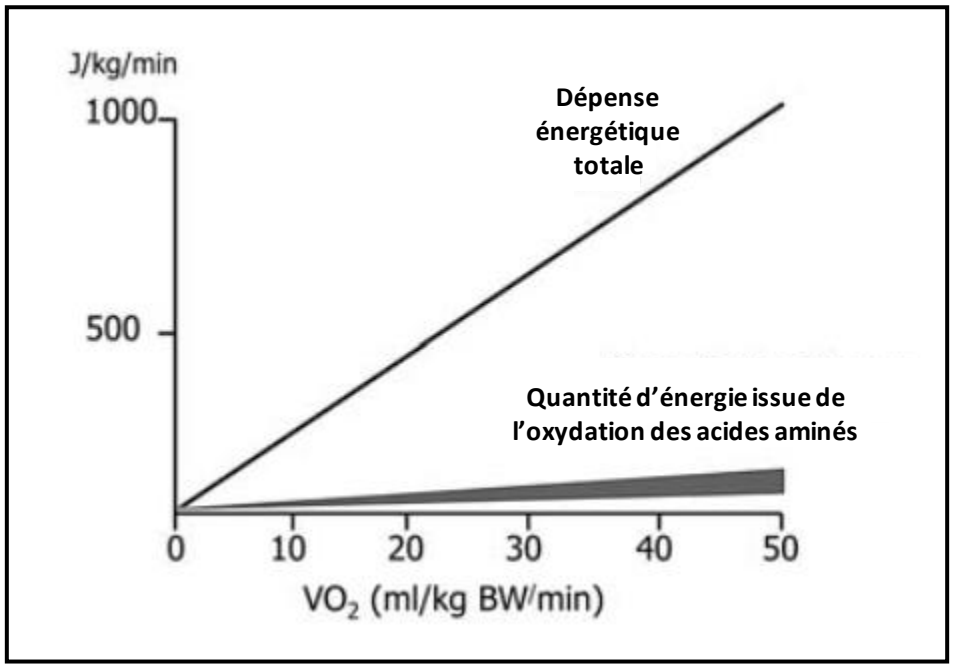

Figure 7. Graphique représentant la quantité d'énergie issue de l'oxydation des protéines à différentes intensités d'exercice

La dépense énergétique augmente de façon proportionnelle à l'intensité (*i.e.* représentée par la consommation d'oxygène : VO_2) de l'exercice pratiqué. La bande grisée représente la contribution de l'oxydation des acides aminés contenus dans les protéines à la dépense énergétique totale. Adapté de (Rennie *et al.*, 2006).

En résumé, différentes voies de production d'ATP existent au sein du muscle squelettique et sont mobilisées de façon adaptée aux besoins énergétiques. Il est important de noter qu'au repos, l'énergie nécessaire au fonctionnement musculaire est presque exclusivement produite de façon aérobie.

Par ailleurs, et de façon intéressante, les fibres musculaires étant de nature hétérogène, le métabolisme énergétique décrit précédemment n'est pas identique dans toutes les fibres au sein d'un même muscle. Différents types de fibres existent, et présentent des caractéristiques fonctionnelles et métaboliques spécifiques (Egan and Zierath, 2013; Monod and Flandrois, 1990; Wilmore and Costill, 2002) (Tableau 1).

Les fibres de type I (*i.e.* « fibres lentes », exprimant l'isoforme de myosine MyHC-1) sont caractérisées par leur couleur rouge (*i.e.* forte concentration de myoglobine) et possèdent une vascularisation importante, une grande quantité de mitochondries, d'enzymes impliquées dans la synthèse aérobie de l'ATP et de réserves lipidiques. Ces fibres disposent donc d'une importante capacité oxydative, mais ne développent pas une grande force de contraction. Ces propriétés leur permettent au repos d'assurer le maintien du tonus musculaire et à l'exercice de soutenir un effort d'intensité modérée et de longue durée (*i.e.* exercice d'endurance).

Les fibres de type II, de couleur rose/blanc (*i.e.* faible concentration de myoglobine), synthétisent majoritairement de l'ATP de façon anaérobie. Les motoneurones innervant les fibres de type II ont un corps cellulaire volumineux et un axone de plus gros diamètre que ceux innervant les fibres de type I. De plus, le nombre de fibres innervées par un seul motoneurone dans une unité motrice (*i.e.* ensemble formé par un motoneurone et les fibres musculaires qu'il innerve) est plus important pour les fibres de type II, c'est la raison pour laquelle ces dernières produisent une force plus importante que les fibres de type I. Deux catégories de fibres de type II existent : les fibres de type IIa (*i.e.* « fibres intermédiaires », exprimant l'isoforme de myosine MyHC-2A) et les fibres de type IIb/IIx (*i.e.* « fibres rapides », exprimant l'isoforme de myosine MyHC-2B ou MyHC-2X). Les fibres de type IIb et IIx possèdent dans l'ensemble des propriétés fonctionnelles et métaboliques similaires, la différence principale résidant dans le fait que les fibres IIb sont retrouvées chez le rongeur et les fibres IIx chez l'homme. Les fibres de type

IIb/IIx possèdent des réserves de glycogène et de phosphocréatine, et synthétisent de l'ATP presque exclusivement de façon anaérobie (lactique et alactique). Ces fibres ont la capacité d'être très rapidement mobilisées et de développer une force importante, mais se fatiguent très vite ; elles sont adaptées à la pratique d'un exercice très intense et de courte durée. Les fibres de type IIa présentent des propriétés intermédiaires entre les fibres de type I et les fibres de type IIb/IIx. L'ATP y est produit de façon anaérobie (lactique et alactique) mais aussi par des mécanismes aérobie. Ainsi, ces fibres peuvent être rapidement mobilisées, développer une force plus importante que les fibres de type I et se fatiguer moins vite que les fibres de type IIb/IIx.

	FIBRES DE TYPE I	**FIBRES DE TYPE IIa**	**FIBRES DE TYPE IIb/IIx**
CARACTERISTIQUES FONCTIONNELLES			
VITESSE DE CONTRACTION	X	XX	XXX
FORCE DEVELOPPEE	X	XX	XXX
RESISTANCE A LA FATIGUE	XXX	XX	X
CARACTERISTIQUES STRUCTURALES			
CONTENU EN MITOCHONDRIES	XXX	XX	X
VASCULARISATION	XXX	XX	X
DIAMETRE DES FIBRES	X	XX	XXX
CARACTERISTIQUES METABOLIQUES			
CONTENU EN GLYCOGENE	X	XX	XXX
CONTENU EN LIPIDES	XXX	XX	X
CAPACITE GLYCOLYTIQUE	X	XX	XXX
CAPACITE OXYDATIVE	XXX	XX	X
VOIES DE PRODUCTION D'ENERGIE			
ANAEROBIE ALACTIQUE	X	XX	XXX
ANAEROBIE LACTIQUE	X	XX	XXX
AEROBIE	XXX	XX	X

Tableau 1. Caractéristiques des différents types de fibres musculaires

Le muscle squelettique est composé de fibres lentes (*i.e.* de type I) et de fibres rapides (*i.e.* de type IIa et IIb/IIx) dotées de caractéristiques fonctionnelles, structurales et métaboliques différentes.

Il est important de noter que chaque muscle possède les trois types de fibres décrits ci-dessus, mais en proportions différentes de façon adaptée à leur fonction.

2.3. ADAPTATIONS MUSCULAIRES A L'EXERCICE PHYSIQUE

La pratique d'une activité physique entraîne une augmentation drastique de la dépense énergétique et l'énergie chimique sous forme d'ATP doit être rapidement produite afin de combler les besoins nécessaires à la contraction musculaire. Il convient toutefois de distinguer deux grandes catégories d'exercices physiques : l'exercice de type aérobie (*i.*e. endurance, intensité modérée à forte) d'une part, et l'exercice de type résistance (*i.e.* sprint, très forte intensité) d'autre part.

Lors de la pratique d'un exercice d'intensité modérée, l'ATP va être fourni principalement par l'oxydation de substrats énergétiques circulants (*i.e.* AG et glucose). Plus l'intensité de l'exercice augmente et (1) plus l'utilisation des substrats énergétique d'origine musculaire est privilégiée (*i.e.* glycogène et IMTG) et (2) plus le métabolisme est orienté vers l'utilisation des glucides (Romijn *et al.*, 1993; Romijn *et al.*, 2000). Il est important de noter qu'à quantité égale, les glucides apportent moins d'énergie que les lipides mais que leur dégradation nécessite moins d'oxygène, les rendant ainsi plus adaptés à une augmentation rapide des besoins en ATP. Ce sont donc les substrats préférentiellement utilisés lors d'un exercice intense. Ainsi, lors de la pratique d'un exercice de forte intensité, la majorité de l'énergie provient de la dégradation des réserves de glycogène musculaire. Cependant, lorsque la durée de l'exercice augmente, la part des lipides oxydés va elle aussi augmenter pour fournir l'énergie nécessaire à la contraction. Notons qu'il existe, pour chaque individu, une intensité d'exercice à laquelle une quantité maximale de lipides va être oxydée, appelée « lipoxmax », correspondant à un exercice pratiqué à une intensité représentant environ 25 à 30 % de la consommation maximale d'oxygène (VO_2max) chez le sujet obèse et pouvant atteindre 65% de la VO_2max chez le sujet entraîné (Horowitz and Klein, 2000). Enfin, les réserves d'IMTG sont préférentiellement utilisées lors d'exercice d'intensité modérée et de longue durée (figure 8).

Lors de la pratique d'un exercice très intense, représentant 4 à 4,5 fois la puissance maximale aérobie (PMA), dont la durée ne peut excéder 10 secondes, les sources d'énergie musculaire sont la phosphocréatine et les réserves d'ATP

musculaires. Lors de la pratique d'un exercice de résistance, représentant 1 à 1,5 fois la PMA et de courte durée, le supplément d'énergie nécessaire à la réalisation de l'exercice est assuré de façon anaérobie par la dégradation des réserves de glycogène conduisant à la production de lactate (Gaitanos *et al.*, 1993; Parolin *et al.*, 1999). Notons que la pratique d'un exercice de type intermittent (*i.e.* alternance pendant une plus longue durée de phases d'exercice de résistance et de repos) entraîne également l'utilisation des réserves d'IMTG, spécifiquement dans les fibres de type I, qui vont être oxydés pour fournir de l'ATP (Koopman *et al.*, 2006).

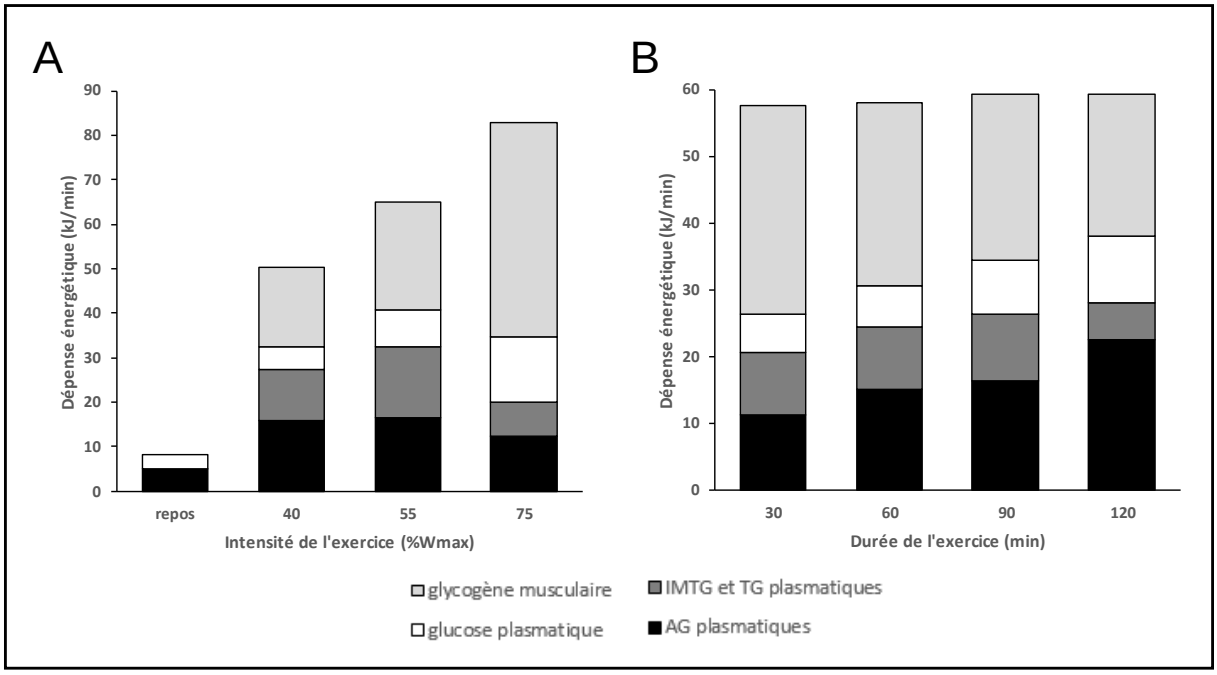

Figure 8. Utilisation des différents substrats énergétiques en fonction de l'intensité et de la durée de l'exercice

(A) Avec l'augmentation de l'intensité de l'exercice il y a une augmentation de l'utilisation des triacylglycérols intramyocellulaires (IMTG) et des acides gras (AG) plasmatiques. Notons que, bien que l'utilisation des lipides soit majoritaire jusqu'à des intensités d'exercice modérées, l'augmentation de la dépense énergétique avec l'intensité est également associée à une augmentation de l'utilisation du glycogène musculaire. En revanche, lors d'un exercice de très forte intensité, les glucides (*i.e.* glucose plasmatique et glycogène musculaire) sont les substrats majoritairement utilisés. (B) Avec la durée de l'exercice (*i.e.* 120min à 50% de l'intensité maximale Wmax), il y a une augmentation de l'utilisation des AG plasmatiques au détriment des IMTG. De même, la part de glucose plasmatique utilisé augmente et celle du glycogène musculaire diminue. Adapté de (van Loon, 2004) et de (van Loon *et al.*, 2003)

Après une session d'exercice, la dépense énergétique diminue fortement mais reste tout de même plus élevée qu'au repos pendant plusieurs heures (Borsheim and Bahr, 2003; Jamurtas *et al.*, 2004). Pendant cette période de récupération, la priorité est attribuée à la reconstitution des réserves de glycogène musculaire. Les lipides (*i.e.* AG circulants) sont alors davantage oxydés pour fournir de l'énergie nécessaire à ce métabolisme (Kuo *et al.*, 2005; Pillard *et al.*, 2010). Notons également que, dans les minutes suivant l'arrêt de l'exercice, les stocks d'ATP et de phosphocréatine sont également reconstitués grâce aux processus oxydatifs. De plus, lors de cette phase de récupération, le lactate accumulé au cours de la session d'exercice va soit être transformé en glucose (*i.e.* gluconéogenèse), soit être oxydé. Rappelons ici que le lactate joue un rôle important dans la protection contre l'apparition d'une acidose musculaire lors de la pratique d'un exercice de forte intensité, en tamponnant l'excès de protons H^+ produits par l'hydrolyse des molécules d'ATP (Robergs *et al.*, 2004). Enfin, l'arrêt de l'exercice entraîne une diminution du catabolisme des protéines en faveur d'une augmentation de leur anabolisme. Les acides aminés vont ainsi être utilisés en post-exercice pour synthétiser des protéines musculaires afin de réparer les fibres endommagées suite à une session d'exercice d'une part, et de permettre l'hypertrophie musculaire induite par un protocole d'entraînement d'autre part (Rennie *et al.*, 2006; Rennie and Tipton, 2000).

Au-delà des modifications du métabolisme musculaire induites par une session d'exercice, il est bien établi que la pratique d'un entraînement physique en endurance s'accompagne de nombreuses adaptations métaboliques et structurales au niveau du muscle squelettique (Egan and Zierath, 2013; Hawley *et al.*, 2014). Les individus entraînés présentent une augmentation de la vascularisation musculaire permettant un meilleur apport de substrats énergétiques et une élimination facilitée des déchets métaboliques produits lors de l'exercice, une hypertrophie des fibres ainsi qu'une augmentation du potentiel oxydatif de ces dernières. De façon intéressante, ces individus présentent une plus grande capacité à oxyder les lipides que des sujets sédentaires et possèdent des réserves de glycogène musculaire et d'IMTG plus importantes. D'un point de vue moléculaire, ces adaptations sont associées à une biogenèse mitochondriale, une augmentation des enzymes impliquées dans l'oxydation des substrats énergétiques et dans la synthèse de glycogène, ainsi qu'à une activation des facteurs de transcription régulant ces

processus (*i.e.* PGC1-α). Par ailleurs, de nouvelles modalités d'entraînement ont vu le jour, du type *high-intensity interval training*, ou HIIT, dont le *sprint interval training* (SIT) fait partie mais qui comprend également de nombreuses autres stratégies d'exercice pratiqué à différentes intensités et pendant des durées plus ou moins longues (Gibala and Little, 2010). Il a été clairement démontré que le HIIT entraîne des améliorations métaboliques importantes sans que la dépense énergétique ne soit considérablement augmentée, mettant ainsi en évidence que cette dernière n'est pas la seule responsable des effets bénéfiques induits par l'entraînement (Burgomaster *et al.*, 2008; Gibala *et al.*, 2006; Gillen *et al.*, 2016; Hwang *et al.*, 2011; Little *et al.*, 2011). Enfin, et de façon importante, l'entraînement permet, au-delà des améliorations musculaires, d'apporter d'importants bénéfices au niveau systémique (*i.e.* cardiovasculaires et métaboliques) (Hawley *et al.*, 2014). Ces améliorations, mettant en jeu de nombreux mécanismes, pourraient être en partie médiées par la capacité du muscle squelettique à sécréter des molécules (*i.e.* myokines) pouvant agir à distance sur d'autres organes, relayant ainsi les effets bénéfiques de l'exercice à l'ensemble de l'organisme (Pedersen and Febbraio, 2012).

3. TISSU ADIPEUX INTRAMUSCULAIRE ET INSULINO-RESISTANCE

De nombreux travaux ont mis en évidence une corrélation positive entre le contenu en IMAT et (i) la taille des autres dépôts adipeux (Boettcher *et al.*, 2009), (ii) une perte de la fonction musculaire (Addison *et al.*, 2014; Tuttle *et al.*, 2012) et (iii) l'insulino-résistance musculaire (Goodpaster *et al.*, 2003; Goodpaster *et al.*, 2000; Yim *et al.*, 2007). Cependant, les mécanismes à l'origine de l'émergence de ces adipocytes intramusculaires sont encore mal compris, tout comme leur potentiel rôle dans le développement d'une insulino-résistance musculaire.

3.1. MECANISMES A L'ORIGINE DE L'ACCUMULATION DE TISSU ADIPEUX INTRAMUSCULAIRE

Une infiltration de lipides, sous formes de gouttelettes dans différents tissus, dont le muscle squelettique, a été décrite pour la première fois en 1965 par W. Aherne chez des enfants nouveau-nés (Aherne, 1965). Quelques années plus tard, en 1976, R. Kannan fut le premier à isoler un dépôt adipeux au sein du muscle squelettique chez la souris, mettant ainsi en évidence pour la première fois l'existence de l'IMAT (Kannan and Baker, 1976; Kannan *et al.*, 1976). Par la suite, la quantification de la présence d'IMAT s'est affinée et sa présence est classiquement mesurée de façon indirecte par tomographie informatisée (CT-scan) ou de façon directe par imagerie à résonance magnétique nucléaire (Addison *et al.*, 2014; Mercuri *et al.*, 2007; Wattjes *et al.*, 2010).

De façon intéressante, une accumulation d'IMAT a été décrite à la fois (i) chez des individus obèses (Hilton *et al.*, 2008), mais également (ii) chez des sujets présentant des pathologies associées à une perte de fonction musculaire comme les personnes atteintes de la dystrophie musculaire de Duchenne (Gaeta *et al.*, 2012; Torriani *et al.*, 2012), ou les sujets âgés atteints de sarcopénie (*i.e.* diminution de la masse musculaire) (Vettor *et al.*, 2009). Dans ce contexte, mieux comprendre les mécanismes à l'origine de l'accumulation d'adipocytes musculaires semble essentiel.

De nombreuses hypothèses ont été proposées pour expliquer les mécanismes à l'origine de l'accumulation d'IMAT. L'hypothèse la plus communément admise à l'heure actuelle consiste en la présence, et la différenciation, de progéniteurs adipocytaires (*fibro/adipogenic progenitors* ou FAP) au sein du muscle squelettique. Ces cellules progénitrices ont été identifiées en 2009 chez l'homme et caractérisées par leur activité aldehyde deshydrogénase et la présence du cluster de différenciation 34 (CD34) à leur surface (Vauchez *et al.*, 2009). De plus, deux autres papiers ont mis en évidence en 2010 que les progéniteurs adipocytaires (*i.e.* FAP) expriment le marqueur CD15 (et sont dépourvues du CD56, marqueur caractéristique des progéniteurs myocytaires chez l'homme, *i.e.* les cellules satellites (Mauro, 1961)) (Lecourt *et al.*, 2010; Pisani *et al.*, 2010b). Ces travaux ont démontré que, lorsqu'ils sont cultivés en conditions adipogéniques, les FAP se différencient en adipocytes. De plus, ces cellules proviennent d'un lignage différent des cellules satellites, et ne semblent pas capables de se différencier en fibres musculaires. La même année, les FAP ont également été identifiées chez la souris. Ces cellules, capables de se différencier en adipocytes et en fibrobastes *in vitro* et *in vivo*, sont caractérisées chez cet animal par la présence à leur surface du *platelet-derived growth factor receptor, alpha polypeptide* (PDGFRα) et du *stem cells antigen-1* (Sca-1) et sont dépourvues de l'Intégrine-α7 (marqueur des cellules satellites) (Uezumi *et al.*, 2010).

De façon surprenante, ces deux groupes de recherche ont décrit que ces cellules sont activées suite à une lésion musculaire induite chez la souris, suggérant ainsi qu'elles puissent jouer un rôle important dans un contexte de régénération musculaire (Joe *et al.*, 2010; Uezumi *et al.*, 2010). De façon intéressante, il a été mis en évidence dans des expériences de co-culture que les FAP favorisent la différenciation des progéniteurs musculaires (Joe *et al.*, 2010), mais que la différenciation adipogénique des FAP est inhibée par la présence de fibres musculaires (Uezumi *et al.*, 2010). Ce dialogue entre FAP et cellules musculaires semble donc être essentiel dans la régulation de l'homéostasie musculaire. Plusieurs facteurs ont été décrits comme pouvant moduler la prolifération et la différenciation de ces progéniteurs adipocytaires. En effet, il a été mis en évidence que l'interleukine-4, dont les taux sont modulés par la présence d'éosinophiles lors d'une lésion musculaire ou par un traitement aux glucocorticoïdes, a un effet positif sur la prolifération des FAP et inhibe leur différenciation adipocytaire (Dong *et al.*, 2014;

Heredia *et al.*, 2013). En revanche, lorsque le processus de régénération est altéré, les FAP se différencient en adipocytes et fibroblastes, entraînant ainsi une perte de la fonction musculaire (figure 9). Il est à noter que le niveau circulant d'interleukine-4 est augmenté avec l'obésité, et ne peut donc pas expliquer la différenciation excessive des FAP observée dans ce contexte (Schmidt *et al.*, 2015). De nombreux autres acteurs moléculaires ont été impliqués dans la différenciation adipocytaire au sein du muscle squelettique (Sciorati *et al.*, 2015). Il a notamment été décrit que le monoxyde d'azote (NO) est capable d'inhiber la différenciation adipocytaire des FAP (Cordani *et al.*, 2014), et que les patients atteints de dystrophie musculaire présentent une altération de l'activité de la NO synthase dans le muscle, associée à une grande quantité d'IMAT (Brenman *et al.*, 1995). Cependant, l'obésité s'accompagne d'une augmentation importante des niveaux musculaires de NO (Eghbalzadeh *et al.*, 2014), rendant peu probable son implication dans l'accumulation d'IMAT observée chez des individus obèses. Il semble donc qu'aucun facteur régulant la différenciation adipocytaire des FAP, parmi ceux identifiés à l'heure actuelle, ne puisse expliquer l'accumulation d'IMAT observée avec l'obésité. Il est toutefois possible d'imaginer que, étant donné que les acides gras (AG) constituent des ligands endogènes du facteur de transcription *peroxysome proliferator-activated receptor γ* (PPARγ) (Marion-Letellier *et al.*, 2015), et que ce dernier est connu pour entraîner la différenciation adipogénique des progéniteurs adipocytaires du tissu adipeux (Lefterova *et al.*, 2014), l'augmentation du niveau d'AG circulants chez des individus obèses (Golay *et al.*, 1986; Jensen *et al.*, 1989; Opie and Walfish, 1963) puisse conduire à l'activation de PPARγ dans les FAP, induisant ainsi leur différenciation accrue en adipocytes dans ce contexte.

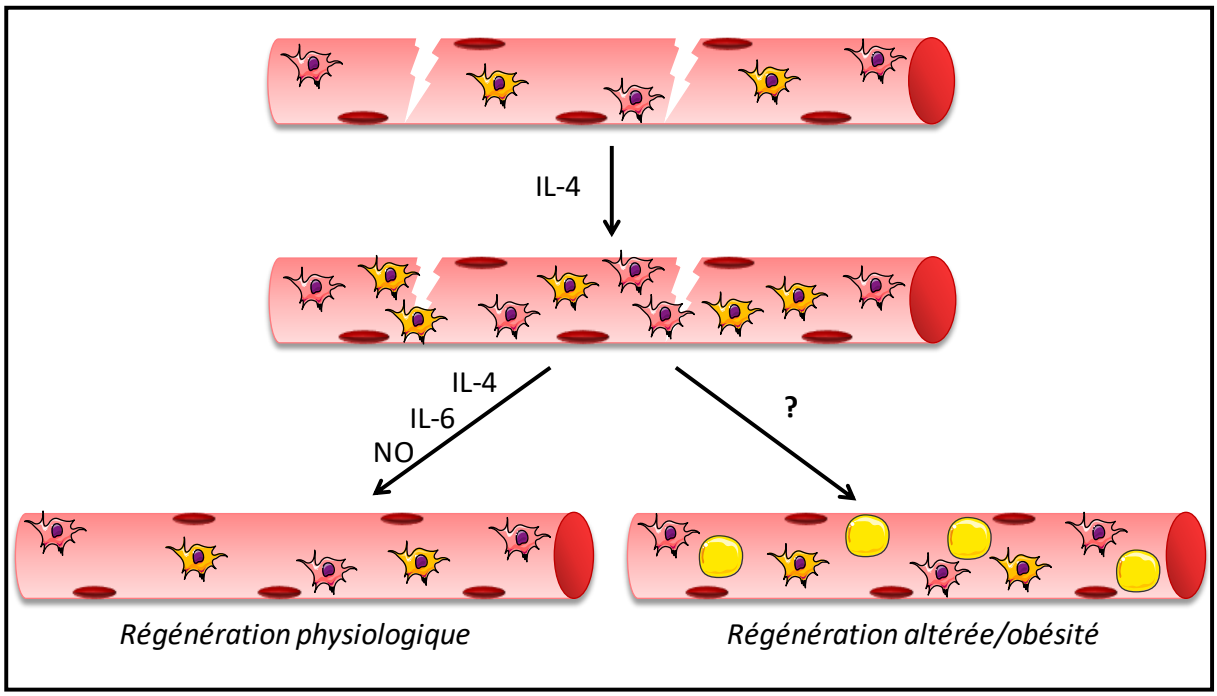

Figure 9. Facteurs influençant la prolifération et la différenciation des FAP impliqués dans la régénération musculaire ou l'accumulation excessive d'IMAT

Lors d'une lésion musculaire, les cellules satellites (en rose) sont activées afin de réparer la fibre endommagée. Les FAP (en orange) prolifèrent et participent activement à la régénération musculaire. Différents facteurs ont été suggérés comme pouvant être impliqués dans l'inhibition de la différenciation des FAP en adipocytes conduisant à une régénération normale. Cependant, les facteurs impliqués dans la dégénération musculaire et/ou l'obésité entraînant l'accumulation d'IMAT n'ont pas été clairement identifiés. IL-4 : interleukine-4 ; IL-6 : interleukine-6 ; NO : monoxyde d'azote

Enfin et de façon intéressante, il a été mis en évidence chez des souris que, suite à une lésion musculaire induite par une injection de glycérol, connue pour entrainer une accumulation d'adipocytes dans le muscle (Pisani *et al.*, 2010a), une immobilisation totale permet de prévenir de l'accumulation d'IMAT. Il semble donc que, dans un contexte de régénération musculaire, l'émergence d'IMAT soit également dépendante des contraintes mécaniques subies par le muscle (Pagano *et al.*, 2015).

D'autres hypothèses ont été proposées pour expliquer l'origine de l'IMAT, parmi lesquelles la trans-différenciation des cellules satellites en adipocytes. En effet, il a été décrit que les cellules satellites sont capables de se différencier non seulement en fibres musculaires, mais également en ostéoblastes, cellules musculaires lisses et adipocytes (Asakura *et al.*, 2001; De Coppi *et al.*, 2006; Shefer *et al.*, 2004). De nombreuses études sur la trans-différenciation des cellules satellites en adipocytes ont été menées *in vitro* sur des cellules musculaires immortalisées (notamment les lignées cellulaires murines) (Hu *et al.*, 1995; Salehzada *et al.*, 2009) ainsi que sur des cellules satellites primaires murines (Asakura *et al.*, 2001) ou dans des fibres musculaires isolées maintenues en culture (De Coppi *et al.*, 2006; Shefer *et al.*, 2004). Cependant, cette capacité de trans-différenciation des cellules satellites a été remise en question et est toujours débattue. En effet, les modèles utilisés sont critiquables, non seulement par les biais que peuvent induire l'usage de lignées cellulaires, mais également par le mode d'isolement des cellules satellites primaires issues de prélèvements musculaires. En effet, les cellules satellites isolées par digestion enzymatique et cultivées en conditions myogéniques peuvent être contaminées par d'autres types cellulaires. Le tri des cellules en cytométrie de flux par FACS (*fluorescence-activated cell sorting*) limite cette contamination par des cellules non-myogéniques (tels que les FAP), mais le choix des marqueurs de surface utilisés est déterminant. Enfin, les études menées sur des fibres musculaires isolées maintenues en culture ont été remises en question lorsqu'il a été mis en évidence que les cellules satellites ne sont pas les seules cellules progénitrices associées aux fibres musculaires, et que des progéniteurs adipocytaires étaient aussi présents dans ces cultures (Chen *et al.*, 2005; Starkey *et al.*, 2011). Ces travaux ont démontré que les cellules exprimant Pax7 (marqueur des cellules satellites quiescentes) ne présentaient en culture qu'un potentiel de différenciation myogénique. De plus, des études de lignage utilisant le système de recombinaison Cre/LoxP ont permis de démontrer chez la souris que les adipocytes intramusculaires sont issus de progéniteurs Pax3 négatifs (marqueur exprimé pendant le développement musculaire), et non des cellules satellites (Liu *et al.*, 2012).

Enfin, nous ne pouvons pas exclure le fait que ces progéniteurs adipocytaires puissent provenir de progéniteurs circulants qui pourraient s'infiltrer dans le muscle squelettique. En effet, il est connu que des cellules circulantes, comme les cellules mésenchymateuses de la moelle osseuse ou les fibrocytes sont capables de s'infiltrer dans différents organes (Chapel *et al.*, 2003; Falkenham *et al.*, 2013; Sener and Albeniz, 2015) et présentent, comme les FAP, un potentiel de différenciation adipogénique (Kokabu *et al.*, 2016). De plus, il a été décrit que des cellules progénitrices du tissu adipeux (ASC, *adipose stromal cells*) sont capables d'une part d'être mobilisées et quitter le tissu adipeux *in vivo* afin de rejoindre la circulation lymphatique (Gil-Ortega *et al.*, 2013), et d'autre part que ces ASC peuvent migrer dans le muscle squelettique lorsqu'elles sont injectées dans la circulation sanguine pour se différencier en cellules musculaires (Forcales, 2015; Liu *et al.*, 2007b; Miura *et al.*, 2008). Etant donné que l'obésité se caractérise par un développement important du tissu adipeux, associé à une accumulation d'IMAT (Boettcher *et al.*, 2009), nous pouvons émettre l'hypothèse que des ASC puissent dans ce cadre sortir du tissu adipeux, s'infiltrer dans le muscle squelettique et se différencier en adipocytes.

En résumé, bien que des progéniteurs adipocytaires aient été identifiés au sein du muscle squelettique, les facteurs influençant leur différenciation et conduisant à l'accumulation d'IMAT sont encore très mal compris et devront faire l'objet de futurs travaux de recherche.

3.2. ASSOCIATION ENTRE ACCUMULATION DE TISSU ADIPEUX INTRAMUSCULAIRE ET INSULINO-RESISTANCE

Une accumulation d'IMAT a été observée chez des sujets diabétiques de type 2 par rapport à des sujets sains (Gallagher *et al.*, 2009), et de nombreuses études décrivent une corrélation positive entre accumulation d'IMAT et insulino-résistance chez l'homme (Boettcher *et al.*, 2009; Goodpaster *et al.*, 2000; Ryan and Nicklas, 1999; Yim *et al.*, 2007). De façon intéressante, même lorsque l'indice de masse corporelle (IMC) est pris en compte dans les calculs, l'IMAT reste un prédicteur important de la glycémie et de l'insulinémie à jeun (Goodpaster *et al.*, 2003). Par ailleurs, une accumulation d'IMAT est également observée chez des sujets âgés

et/ou sédentaires (Marcus *et al.*, 2010). Une étude longitudinale menée chez des jumeaux a démontré qu'après 32 ans de divergence dans la pratique d'activité physique, les individus inactifs présentaient 54% d'IMAT en plus que leur jumeau pratiquant une activité physique régulière (Leskinen *et al.*, 2009). Par ailleurs, des travaux de l'équipe de B. Goodpaster ont montré que la pratique d'une activité physique régulière prévient de l'accumulation d'IMAT associée à l'âge (Goodpaster *et al.*, 2008; Wroblewski *et al.*, 2011). De plus, la perte de poids induite par un régime hypocalorique permet également de réduire la quantité d'IMAT (Christiansen *et al.*, 2009). De façon intéressante, de nombreuses études ont montré qu'une perte de poids induite par la pratique d'une activité physique réduirait davantage la quantité d'IMAT qu'une perte de poids induite uniquement par la restriction calorique (Avila *et al.*, 2010; Murphy *et al.*, 2012). Il a ainsi été suggéré que, chez des individus entraînés, lors de la pratique d'un exercice physique l'IMAT pourrait servir de substrat afin de fournir de l'énergie aux cellules musculaires (Prior *et al.*, 2007), bien qu'aucune étude n'ait à l'heure actuelle évalué la validité de cette hypothèse. Il est connu que l'activité physique et/ou la perte de poids ont de nombreux effets bénéfiques sur le métabolisme, et l'implication spécifique de la diminution d'IMAT dans ce contexte n'a pas été étudiée, les travaux réalisés jusqu'à présent étant uniquement corrélatifs.

Ainsi, le rôle d'une accumulation d'IMAT comme évènement causal ou simple marqueur d'une insulino-résistance systémique n'a pas été clairement établi à ce jour. Considérant la proximité physique entre IMAT et fibres musculaires, nous pouvons émettre l'hypothèse que les adipocytes intramusculaires puissent envoyer des signaux entrainant de façon paracrine une altération du métabolisme des fibres musculaires qui les entourent (cytokines inflammatoires, médiateurs lipidiques…). Il a par exemple été démontré que, chez des sujets ayant survécu à un accident vasculaire cérébral, la quantité d'IMAT ainsi que l'inflammation musculaire étaient augmentées dans la jambe parétique, suggérant ainsi que l'IMAT puisse sécréter des cytokines pro-inflammatoires, bien qu'aucun lien direct n'ait été mis en évidence (Hafer-Macko *et al.*, 2005; Ryan *et al.*, 2011). L'existence d'un dialogue entre adipocytes et cellules musculaires pouvant altérer la sensibilité à l'insuline a toutefois été démontré *in vitro* dans des lignées cellulaires murines. La co-culture d'adipocytes 3T3-L1 avec des myotubes L6 (*i.e.* cellules musculaires différenciées en culture)

entraîne une sécrétion d'interleukine-6 et une altération de la signalisation insulinique dans les myotubes L6 (Seyoum *et al.*, 2011). Cependant, aucune donnée chez l'homme n'est actuellement disponible pour soutenir l'hypothèse d'un dialogue entre adipocytes intramusculaires et fibres musculaires entraînant une altération de la sensibilité à l'insuline, et c'est ce qui a fait l'objet de la première publication réalisée dans le cadre de ma thèse.

4. TRIACYLGLYCEROLS INTRAMYOCELLULAIRES ET INSULINO-RESISTANCE

Les lipides sont stockés dans les fibres musculaires sous forme de TAG au sein de gouttelettes lipidiques. Ces TAG intramyocellulaires (IMTG) constituent une réserve d'énergie indispensable au bon fonctionnement du muscle, dans laquelle il pourra puiser lorsqu'il en a besoin, par exemple à jeun ou pendant un exercice physique (Kiens, 2006; van Loon, 2004).

4.1. SYNTHESE DE TRIACYLGLYCEROLS INTRAMYOCELLULAIRES

Les IMTG proviennent de l'estérification des AG plasmatiques à l'intérieur des fibres musculaires (figure 10). Les AG circulants sont transportés à l'intérieur des cellules musculaires par les transporteurs CD36 (*cluster of differenciation 36*) et FATP1 (*fatty acid transport protein 1*). Différentes études chez la souris ont montré que les animaux présentant une délétion du gène codant pour les protéines CD36 et FATP1 sont protégées d'une accumulation d'IMTG lorsqu'elles sont nourries avec un régime riche en graisses (Goudriaan *et al.*, 2003; Hajri *et al.*, 2002; Kim *et al.*, 2004). Une fois transportés dans le cytosol, les AG sont activés en acyl-CoA par l'acyl-coA synthétase. La première étape de la synthèse des IMTG est contrôlée par la GPAT (*glycerol-3-phosphate acyltransferase*), qui catalyse la formation d'acide lysophosphatidique (LPA) à partir d'un acyl-CoA et de glycérol 3 phosphate. L'AGPAT (*1-acylglycerol-3-phosphate O-acyltransferase*) va ensuite synthétiser de l'acide phosphatique (PA) à partir du LPA. Le PA est ensuite converti en diacylglycérol (DAG) par la PAP (*PA phosphatase*). Les DAG peuvent aussi provenir des monoacylglyérols (MAG) suite à l'action de la MGAT (*MG acyltransferase*), eux-mêmes synthétisés à partir de glycérol-3-phosphate (G3P) et d'acyl-CoA par la GPAT (G3P acyltransferase) (Coleman *et al.*, 2000; Watt and Hoy, 2012). L'étape finale de la synthèse d'IMTG est catalysée par la DGAT (*DAG acyltransferase*), qui acyle les DAG en TAG. Des surexpressions musculaires de la DGAT1 (Liu *et al.*, 2007a) ou de la DGAT2 (Levin *et al.*, 2007) chez la souris entraînent une accumulation d'IMTG et une diminution de la quantité de DAG.

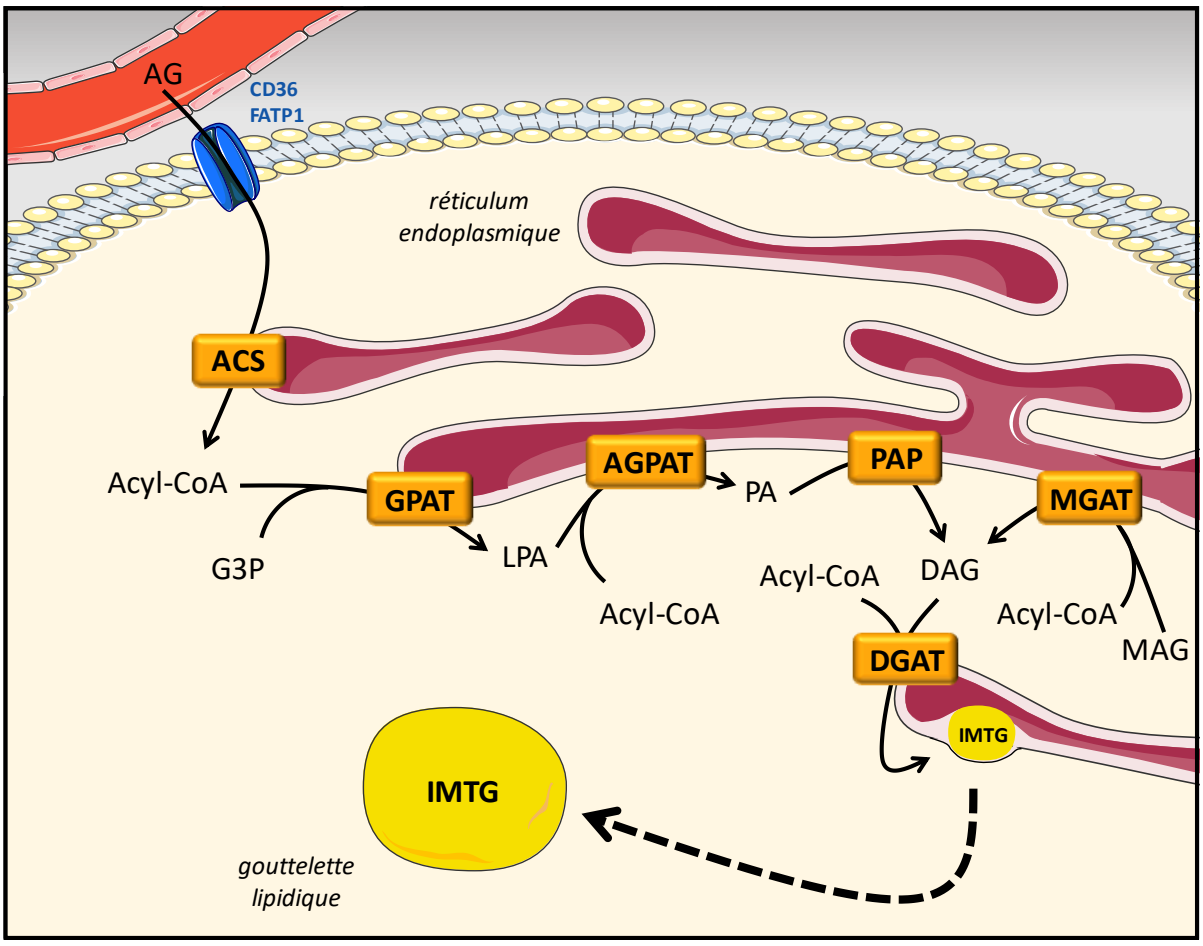

Figure 10. Synthèse des triacylglycérols intramyocellulaires

Les acides gras (AG) présents dans la circulation sanguine sont transportés à l'intérieur des cellules musculaires via le cluster de différenciation 36 (CD36) et *fatty acid transport protein 1* (FATP1). Ces AG sont ensuite activés en acyl-CoA par l'*acyl-CoA synthase* (ACS). L'acide lysophosphatidique (LPA) est ensuite synthétisé par la *glycerol-3-phosphate acyltransferase* (GPAT) à partir d'acyl-CoA et de glycérol-3-phosphate (G3P). La *1-acylglycerol-3-phosphate O-acyltransferase* (AGPAT) forme ensuite de l'acide phosphatidique (PA) à partir du LPA et d'un acyl-CoA. Le PA est transformé en diacylglycérol (DAG) par la *phosphatidic acid phosphatase* (PAP). Les DAG peuvent aussi provenir de l'ajout d'un acyl-CoA à un monoacylglycérol (MAG) par la *monoacylglycerol acyltransferase* (MGAT). L'étape finale de la synthèse des triacylglycérols intramyocellulaires (IMTG) est catalysée par la *diacylglycerol acyltransferase* (DGAT) à partir d'un DAG et d'un acyl-CoA.

4.2. LIEN ENTRE TRIACYLGLYCEROLS INTRAMYOCELLULAIRES ET INSULINO-RESISTANCE

Une augmentation du contenu en IMTG a été observée chez des individus obèses et diabétiques de type 2 par rapport à des sujets sains de poids normal (Moro *et al.*, 2009). Par ailleurs, des études menées chez des individus sains ont reporté que les IMTG sont un meilleur prédicteur de la sensibilité à l'insuline musculaire que ne le sont les AG circulants (Krssak *et al.*, 1999). De façon intéressante, plusieurs études chez l'homme et le rongeur obèses ont montré une hausse de l'entrée des AG dans le muscle squelettique, ce qui pourrait participer à l'augmentation de la quantité d'IMTG observée chez les individus obèses (Bonen *et al.*, 2004; Luiken *et al.*, 2001). Enfin, il a été mis en évidence chez des individus sédentaires une forte corrélation négative entre le contenu en IMTG et la sensibilité à l'insuline systémique (Goodpaster *et al.*, 1997; Jacob *et al.*, 1999; Pan *et al.*, 1997), et cette relation est indépendante de l'IMC (McGarry, 2002).

Cependant, il a également été observé que des athlètes entraînés en endurance ont eux aussi une quantité accrue d'IMTG par rapport à des sujets sains non-entraînés, comparable à celle de sujets obèses/diabétiques, mais sont malgré tout très sensibles à l'action de l'insuline (figure 11). Ce paradoxe a été mis en évidence pour la première fois en 2001, et est désormais connu sous le nom de « paradoxe des athlètes » (Goodpaster *et al.*, 2001). Cette observation à priori contradictoire avec les travaux publiés à l'époque pourrait s'expliquer par une meilleure capacité du muscle squelettique de sujets entraînés à gérer les réserves lipidiques, notamment par un meilleur couplage entre stockage et utilisation des IMTG, les athlètes présentant une meilleure capacité oxydative musculaire que des sujets sédentaires (normo-pondérés ou obèses) (Gollnick *et al.*, 1973; Goodpaster *et al.*, 2001; Jansson and Kaijser, 1987). De façon intéressante, une proximité physique accrue entre les gouttelettes lipidiques et les mitochondries a été reportée chez des sujets actifs par rapport à des sujets sédentaires, ainsi qu'une augmentation du turnover des IMTG (Jansson and Kaijser, 1987; Jong-Yeon *et al.*, 2002; Moro *et al.*, 2008; Sacchetti *et al.*, 2004; Tarnopolsky *et al.*, 2007). Par ailleurs, plusieurs études ont mis en évidence que faire suivre un entraînement en endurance de plusieurs semaines à des sujets normo-pondérés permet d'améliorer leur sensibilité à

l'insuline, et que ceci s'accompagne d'une augmentation du contenu en IMTG (Schrauwen-Hinderling *et al.*, 2003; Shepherd *et al.*, 2013). En revanche, des études chez des sujets obèses ont montré que l'amélioration de la sensibilité à l'insuline induite par un entraînement en endurance était associée soit à une diminution (Louche *et al.*, 2013) soit à une augmentation (Dube *et al.*, 2008; Dube *et al.*, 2011) du contenu en IMTG. Ces différences peuvent s'expliquer par des protocoles d'entraînement différents, des spécificités de populations, des différences dans le choix du moment où les biopsies ont été prélevées en fonction des interventions subies, mais aussi par les techniques de mesure des IMTG pouvant varier d'une étude à l'autre. Par exemple, l'étude menée par J.J. Dubé *et al* a examiné le contenu en IMTG par imagerie en marquant les gouttelettes lipidiques avec des sondes fluorescentes, permettant une quantification spécifique des IMTG (Dube *et al.*, 2008) alors que dans le travail réalisé au sein de notre laboratoire, une approche par spectrométrie de masse a été utilisée pour quantifier les lipides musculaires (Louche *et al.*, 2013).

L'ensemble de ces travaux démontre clairement qu'il existe un découplage entre accumulation d'IMTG et insulino-résistance, suggérant ainsi que les IMTG ne seraient pas responsables *per se* du développement d'une insulino-résistance dans le muscle squelettique

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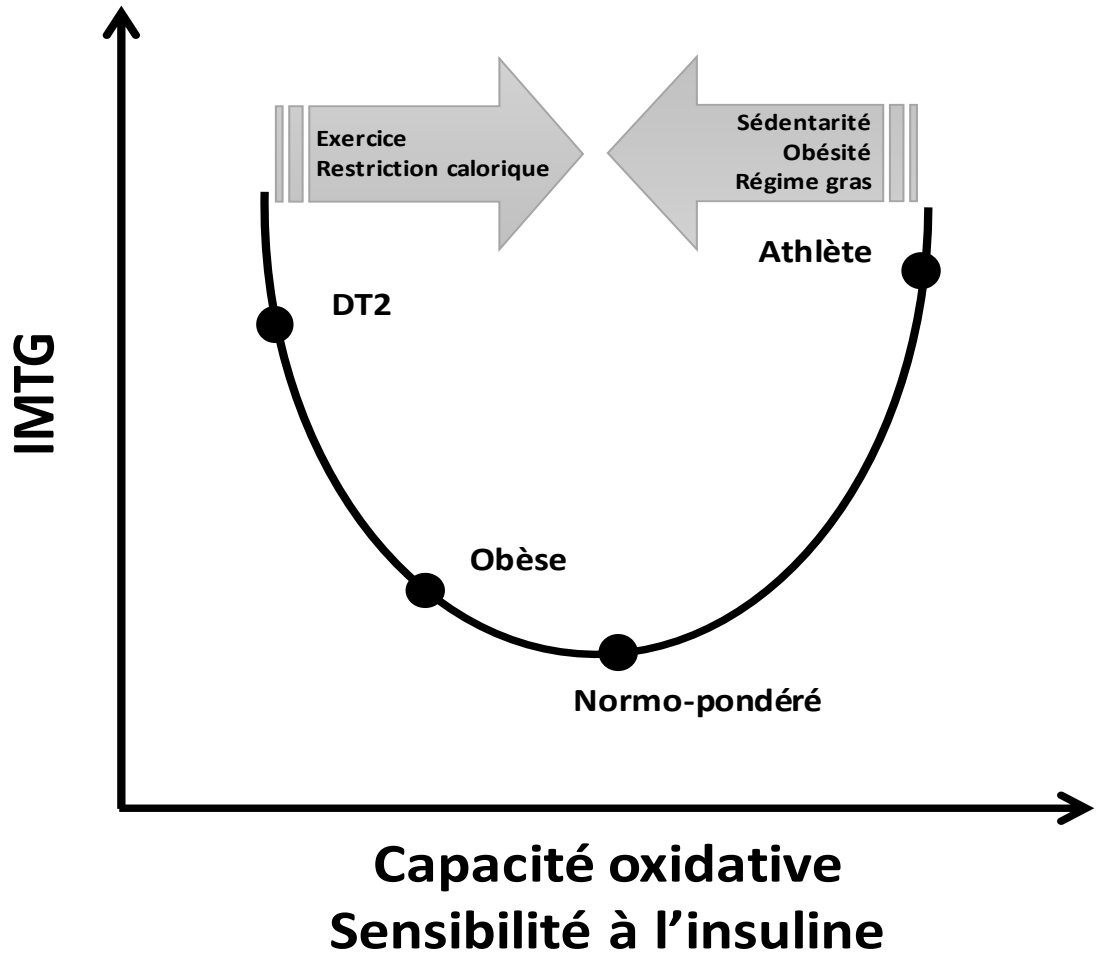

Figure 11. Modèle illustrant le paradoxe des athlètes

Ce modèle représente la relation entre les triacylglycérols intramyocellulaires (IMTG), la capacité oxydative et la sensibilité à l'insuline chez des individus normo-pondérés, obèses, diabétiques de type 2 (DT2) et chez des athlètes, ainsi que certains facteurs pouvant modifier ces paramètres. (Moro *et al.*, 2008)

5. ACCUMULATION D'ESPECES LIPIDIQUES LIPOTOXIQUES ET INSULINO-RESISTANCE MUSCULAIRE

Depuis qu'il a été établi que les IMTG ne sont pas directement responsables du développement de l'insulino-résistance musculaire, de nombreuses études ont cherché à identifier les acteurs moléculaires impliqués dans l'altération de la sensibilité à l'insuline du muscle squelettique. La génération d'espèces actives de l'oxygène induite par une surcharge lipidique au niveau des mitochondries (Pillarisetti and Saxena, 2004), la peroxydation lipidique due à la stagnation des IMTG dans les cellules musculaires (Russell, 2004) ainsi qu'un stress du réticulum endoplasmique (Flamment *et al.*, 2012; Hotamisligil, 2010) ont été associés à l'insulino-résistance musculaire. Bien qu'il soit communément admis que ces mécanismes sont associés à l'insulino-résistance, leur implication en tant que cause ou conséquence de la diminution de la sensibilité à l'insuline est toujours débattu (Boden, 2009; Bonnard *et al.*, 2008; Martinez, 2006; Montgomery and Turner, 2015). Par ailleurs, des espèces lipidiques dérivées des IMTG telles que les acyl-CoA à longue chaîne (ACoA-LC), les diacylglycérols et les céramides ont ainsi été associées à la dérégulation de la signalisation insulinique au sein du muscle squelettique (figure 12).

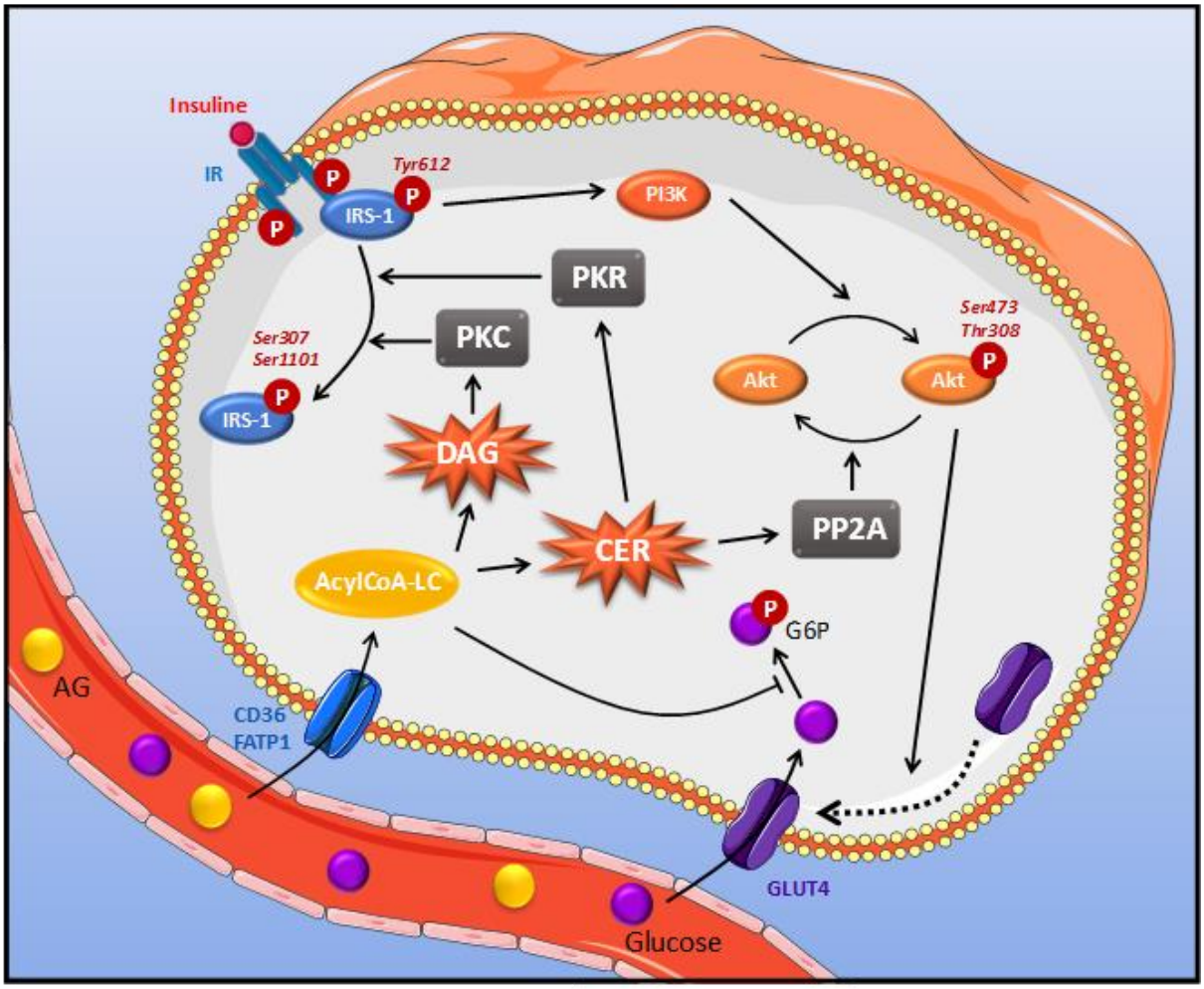

Figure 12. Lipides lipotoxiques et insulino-résistance musculaire

Les Acyl-CoA à longue chaîne (AcylCoA-LC), les diacylglycérols (DAG) et les céramides (CER) sont capables d'inhiber la signalisation insulinique par différents mécanismes. Les AcylCoA-LC sont capables d'inhiber l'hexokinase, responsable de la phosphorylation de glucose en glucose-6-phosphate (G6P), mais aussi de participer à la synthèse de DAG et de CER. Les DAG sont capables d'activer des isoformes de protéine kinase C (PKC) responsables de phosphorylations inhibitrices de l'*insulin receptor substrate 1* (IRS-1) sur des résidus inhibiteurs. Les CER peuvent activer la protéine phosphatase 2A (PP2A) responsable de la déphosphorylation des résidus activateurs d'Akt. Les CER peuvent aussi activer la *double-stranded RNA-activated protein kinase* (PKR), responsable de la phosphorylation d'IRS-1 sur des résidus inhibiteurs. CD36 : cluster de différenciation 36 : FATP1 : *fatty acid transport protein 1* ; GLUT4 : *glucose transporter 4* ; IR : récepteur à l'insuline ; PI3K : phosphatidylinositol 3-kinase

5.1. ACYL-CoA A LONGUE CHAINE

Une augmentation de la quantité musculaire ACoA-LC a été décrite chez le rongeur lors d'un régime hyperlipidique (Chen *et al.*, 1992; Oakes *et al.*, 1997; Song *et al.*, 2012), ainsi que chez des sujets obèses par rapport à des sujets normo-pondérés (Ellis *et al.*, 2000; Hulver *et al.*, 2003). Par ailleurs, des études d'infusion de lipides ont permis de démontrer qu'une élévation du taux d'AG circulants entraîne une accumulation d'ACoA-LC dans le muscle ainsi qu'une détérioration de la sensibilité à l'insuline (Chalkley *et al.*, 1998; Tsintzas *et al.*, 2007).

Afin d'étudier le rôle causal d'une accumulation d'ACoA-LC dans le développement de l'insulino-résistance musculaire, des études ont été réalisées *in vitro* et ont permis de montrer que les ACoA-LC inhibent l'hexokinase musculaire (Thompson and Cooney, 2000). Cette enzyme phosphoryle le glucose en glucose-6-phosphate et a, via le contrôle du gradient de concentration de glucose, un rôle limitant dans son transport musculaire (Fueger, 2005). Le rôle physiologique de l'inhibition de l'hexokinase par les ACoA-LC pourrait être de bloquer de façon transitoire l'entrée du glucose dans les cellules musculaires afin de privilégier l'oxydation des lipides, protégeant ainsi la cellule de leur accumulation cytosolique. Cependant, l'accumulation d'ACoA-LC pourrait conduire à une inhibition chronique de l'hexokinase, conduisant à une diminution du transport musculaire de glucose pouvant expliquer en partie l'altération de l'action de l'insuline induite par les lipides chez des individus obèses (Randle *et al.*, 1965; Thompson and Cooney, 2000).

De plus, il a été montré que des souris invalidées pour la GPAT mitochondriale accumulent des ACoA-LC dans le foie, mais pas de TAG ni de DAG, et sont protégées du développement de l'insulino-résistance induite par un régime gras (Neschen *et al.*, 2005). A l'inverse, des souris surexprimant la GPAT mitochondriale dans le foie n'accumulent pas d'ACoA-LC mais développent une insulino-résistance hépatique associée à une augmentation de DAG et de TAG (Nagle *et al.*, 2007). Ces études suggèrent donc que les ACoA-LC ne sont pas responsables *per se* de l'altération de la sensibilité à l'insuline.

En revanche, il a été démontré que les ACoA-LC entrent dans la synthèse d'autres espèces lipotoxiques, notamment les DAG et les céramides, et pourraient ainsi jouer un rôle indirect dans le développement de l'insulino-résistance.

5.2. DIACYLGLYCEROLS

Une augmentation de la quantité de DAG musculaires a été décrite chez le rongeur nourri avec un régime hyperlipique (Badin *et al.*, 2013; Turner *et al.*, 2013) ainsi que chez l'homme dans le muscle de sujets obèses et/ou insulino-résistants par rapport à des sujets sains (Moro *et al.*, 2009; Straczkowski *et al.*, 2007). De plus, il a été mis en évidence dans des études interventionnelles qu'une amélioration de la sensibilité à l'insuline induite par un entraînement physique et/ou une restriction calorique s'accompagne d'une diminution de la quantité de DAG musculaires (Bruce *et al.*, 2006; Dube *et al.*, 2008; Dube *et al.*, 2011).

Chez le rongeur, des études d'infusion de lipides ayant pour but d'augmenter la concentration d'AG plasmatiques induisent une diminution de la sensibilité à l'insuline dès 3h après le début de l'infusion, concomitante avec une accumulation de DAG, une altération de la signalisation insulinique et du transport de glucose dans le muscle squelettique, sans aucune modification du contenu en IMTG (Griffin *et al.*, 1999; Yu *et al.*, 2002). Une étude d'infusion d'intralipides réalisée en 2002 par S.I. Itani *et al* a permis de démontrer une association entre accumulation de DAG, augmentation de l'activité de protéines kinases C (PKC) et insulino-résistance chez l'homme (Itani *et al.*, 2002). De plus, il a été montré dans des modèles animaux qu'une surexpression musculaire de la DGAT1 entraîne une augmentation de la quantité de TAG, une diminution de la quantité de DAG (Liu *et al.*, 2007a), et permet de protéger le muscle du développement d'une insulino-résistance induite par un régime gras (Timmers *et al.*, 2011).

Il est toutefois important de noter que plusieurs études contradictoires existent, ne montrant aucune modification du contenu en DAG suite à un entraînement physique (Louche *et al.*, 2013), une restriction calorique (Anastasiou *et al.*, 2010) ou chez des sujets obèses/diabétiques par rapport à des sujets sains (Thrush *et al.*, 2009; van Hees *et al.*, 2011). Cependant, hormis les différences dans les modalités expérimentales et les critères de recrutement propres à chaque étude, plusieurs hypothèses peuvent expliquer ces différences. Tout d'abord, les DAG ont été mesurés dans ces études sur des lysats musculaires, ne prenant donc pas en compte leur compartimentation subcellulaire, notamment leur localisation membranaire ou cytosolique. De plus, la structure des DAG (localisation des AG sur

le squelette de glycérol, longueur de chaîne et degré de saturation des AG) semble jouer un rôle important dans leur capacité à activer les PKC et ainsi altérer la signalisation insulinique (Amati, 2012). Il a notamment été mis en évidence que les DAG sont davantage localisés à la membrane plasmique chez des individus obèses/diabétiques par rapport à des athlètes, et que l'activation de PKCε est corrélée positivement à la quantité de DAG membranaires et négativement à la quantité de DAG cytosoliques. De plus, une corrélation avec l'insulino-résistance a uniquement été observée avec les DAG membranaires et les DAG saturés (Di-C18:0) dans cette étude (Bergman *et al.*, 2012). De façon intéressante, il a été mis en évidence que le degré de saturation des DAG musculaires reflète la composition en AG du régime alimentaire (Kien *et al.*, 2011), pouvant expliquer l'accumulation de DAG saturés observée avec l'obésité.

En résumé, même si de nombreuses évidences soulignent une association entre accumulation de DAG et insulino-résistance musculaire, leur rôle causal dans l'altération de la sensibilité à l'insuline a fait débat au sein de la communauté scientifique, et les futurs travaux de recherche dans ce domaine devront s'attacher à étudier la localisation subcellulaire et la composition des DAG, et non mesurer uniquement leur quantité totale.

5.3. CERAMIDES

Plusieurs études ont mis en évidence une association entre l'accumulation musculaire de céramides et le développement de l'insulino-résistance chez le rongeur et chez l'homme (Fillmore *et al.*, 2015; Holloway *et al.*, 2014; Moro *et al.*, 2009; Straczkowski *et al.*, 2004). Il a notamment été mis en évidence une accumulation de céramides dans le muscle de patients obèses et/ou diabétiques par rapport à des sujets sains (Adams *et al.*, 2004; Amati *et al.*, 2011). De plus, des études interventionnelles d'entraînement en endurance ont permis de constater une association entre amélioration de la sensibilité à l'insuline et diminution du contenu en céramides musculaires (Dobrzyn *et al.*, 2004; Dube *et al.*, 2011). Par ailleurs, comme observé pour les DAG, un régime hyperlipidique chez le rongeur et l'infusion d'intralipides chez l'homme induisent une insulino-résistance qui s'accompagne d'une accumulation de céramides dans le muscle squelettique (Blachnio-Zabielska *et*

al., 2010; Frangioudakis *et al.*, 2010; Straczkowski *et al.*, 2004). De façon intéressante, il a été montré que des souris nourries avec un régime riche en graisses sont protégées du développement d'une intolérance au glucose si elles sont traitées avec de la myriocine, un inhibiteur de la *serine palmitoyl transferase 1* (SPT1), enzyme de synthèse des céramides (figure 13), induisant une diminution de la concentration musculaire de céramides (Ussher *et al.*, 2010). Cependant, le rôle des céramides semble être limité au développement d'une insulino-résistance induite par les lipides saturés. Il a en effet été observé que la myriocine prévient du développement d'une insulino-résistance musculaire induite par le palmitate (AG saturé) mais pas par le linoléate (AG insaturé) (Holland *et al.*, 2007; Zierath, 2007). Ces observations pourraient ainsi contribuer à expliquer le lien entre la prise d'un régime riche en AG saturés (« *junk food* ») observée chez des individus obèses (Mistry and Puthussery, 2015), et le développement de l'insulino-résistance.

En résumé, ces études ont permis de démontrer que les céramides jouent un rôle causal dans l'altération de la sensibilité à l'insuline par trois mécanismes distincts, et il serait maintenant intéressant de déterminer la part de chacun dans le développement d'une insulino-résistance chez l'homme.

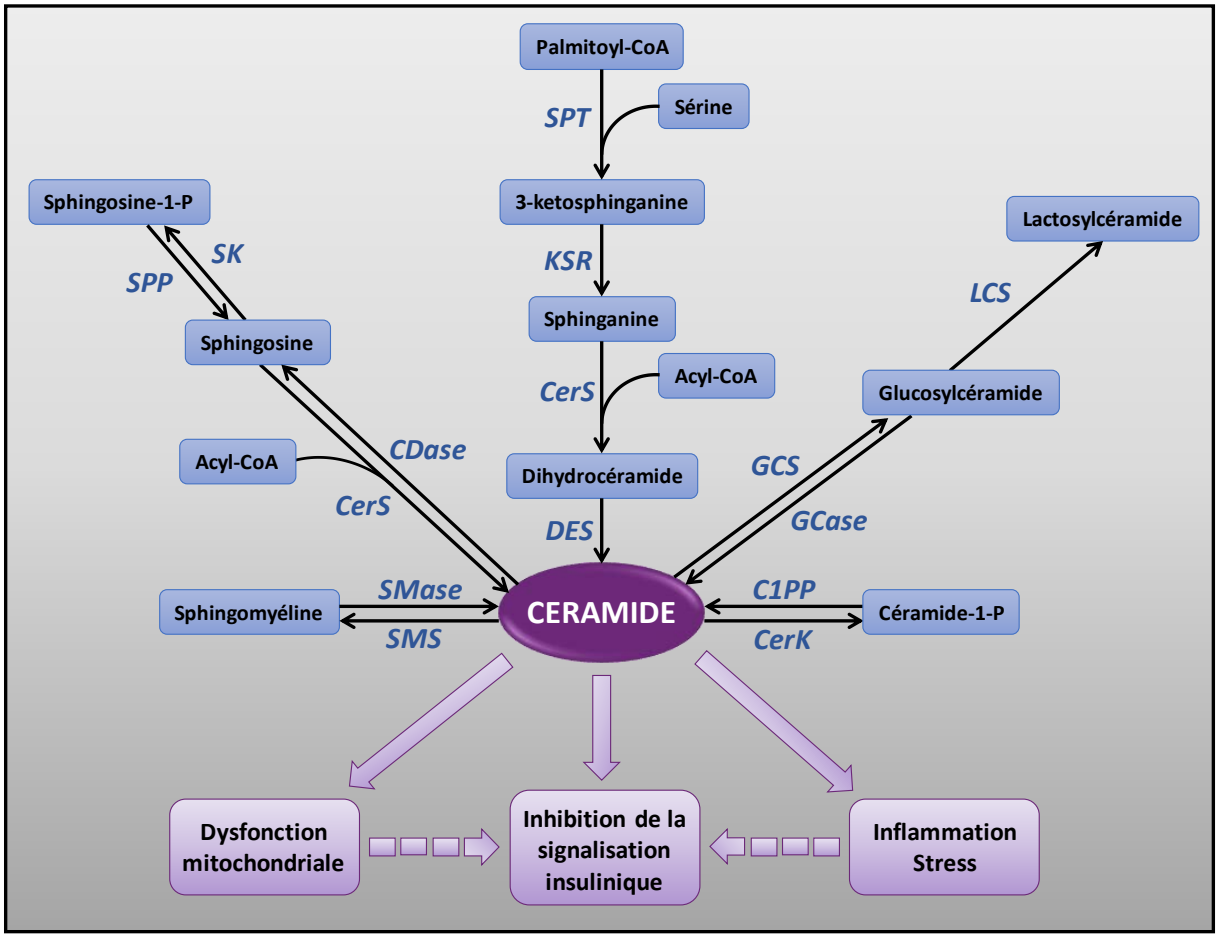

Figure 13. Anabolisme et catabolisme des céramides

Les céramides peuvent être synthétisées à partir de différentes voies, et leur accumulation cytosolique peut conduire à une une dysfonction mitochondriale, un état d'inflammation et de stress cellulaire ainsi qu'à une altération de la signalisation insulinique. SPT : *serine palmitoyltransferase* ; KSR : *3-ketosphinganine reductase* ; CerS : *ceramide synthase* ; DES : *desaturase* ; CDase : *ceramidase* ; SK : *sphingosine kinase* ; SPP : *sphingosine-1-phosphate phosphatase* ; SMase : *sphingomyelinase* ; SMS : *sphingomyeline synthase* ; CerK : *ceramide kinase* ; C1PP : *ceramide-1-phosphate phosphatase* ; GCS : *Glucosylceramide synthase* ; GCase : *glucosylceramidase* ; LCS : *lactosylceramide synthase*

5.4. MECANISMES MOLECULAIRES DE L'ALTERATION DU SIGNAL INSULINIQUE

Les DAG sont connus de longue date comme étant capables d'activer de façon allostérique les PKC conventionnelles (α, βI, βII, γ) et nouvelles (δ, ε, η, θ) (Nishizuka, 1995). Les PKC sont quant à elles capables d'inhiber l'activité d'IRS1 en

le phosphorylant sur des résidus inhibiteurs (ser1101, ser307), expliquant ainsi le lien entre accumulation de DAG et altération de la signalisation insulinique (Li *et al.*, 2004; Ravichandran *et al.*, 2001; Yu *et al.*, 2002). D'autre sites de phosphorylation d'IRS-1 ont été décrits mais sont bien moins documentés et devront faire l'objet de futurs travaux de recherche (Gual *et al.*, 2005; White, 2006).

Par ailleurs, afin de déterminer le rôle causal des céramides dans la dégradation de la sensibilité à l'insuline et d'identifier les mécanismes moléculaires impliqués, des études ont été réalisées *in vitro* sur des cultures cellulaires. Il a ainsi été mis en évidence que les céramides sont capables d'activer la PKCξ (isoforme atypique de PKC), entraînant ainsi une phosphorylation d'Akt sur un résidu inhibiteur (Thr34), empêchant sa translocation à la membrane plasmique induite par l'insuline (Mahfouz *et al.*, 2014; Powell *et al.*, 2003; Powell *et al.*, 2004). Par ailleurs, il a également été montré que les céramides sont aussi capables d'activer la protéine phosphatase 2A (PP2A), connue pour déphosphoryler Akt sur ses résidus activateurs (ser473 et thr308) (Chavez *et al.*, 2003; Teruel *et al.*, 2001). Enfin, une étude récente a démontré que les céramides sont également impliquées dans l'altération de la signalisation insulinique au niveau d'IRS1, en induisant une phosphorylation inactivatrice (ser307) médiée par la PKR (*double-stranded RNA-activated protein kinase*) (Hage Hassan *et al.*, 2016).

5.5. MECANISMES A L'ORIGINE DE L'ACCUMULATION D'ESPECES LIPOTOXIQUES

L'étude de la clairance d'acides gras radiomarqués a permis de mettre en évidence une augmentation de l'entrée des AG dans le muscle squelettique de rats insulino-résistants (Hegarty *et al.*, 2002), ce qui a été également observé chez des individus obèses et/ou diabétiques (Aguer *et al.*, 2010; Bonen *et al.*, 2004). Cette entrée accrue d'AG dans les cellules semble pouvoir être expliquée par deux hypothèses non mutuellement exclusives : l'augmentation du taux d'AG circulants d'une part, et l'augmentation des transporteurs d'AG à la membrane des cellules musculaires d'autre part. En effet, il a été démontré par des études d'infusion de lipides qu'une élévation du niveau d'AG plasmatiques est suffisante pour entraîner l'accumulation musculaire de lipides lipotoxiques induisant le développement d'une

insulino-résistance (Griffin *et al.*, 1999; Straczkowski *et al.*, 2004; Yu *et al.*, 2002). Il est important de noter qu'une augmentation des niveaux circulants d'AG a été observée chez des sujets obèses et diabétiques (Golay *et al.*, 1986; Jensen *et al.*, 1989; Opie and Walfish, 1963), pouvant ainsi contribuer par un effet de flux à l'augmentation du transport musculaire d'acides gras décrite chez ces individus. Par ailleurs, il a été mis en évidence dans des modèles animaux que la délétion des transporteurs musculaires d'AG CD36 et FATP1 entraîne non seulement une diminution de l'entrée des AG dans les cellules musculaires, mais également une diminution de l'accumulation intracellulaire de lipides et une amélioration de la sensibilité à l'insuline musculaire (Bonen *et al.*, 2007; Goudriaan *et al.*, 2003; Kim *et al.*, 2004). De façon intéressante, une augmentation spécifique de la quantité de CD36 à la membrane plasmique, sans modification de son expression protéique totale, a été observée chez des individus obèses et diabétiques (Aguer *et al.*, 2010; Bonen *et al.*, 2004). L'ensemble de ces résultats indique donc que l'augmentation du transport musculaire d'AG observé avec l'obésité pourrait être à l'origine d'une accumulation d'AG intramusculaires et d'espèces lipotoxiques associées.

Par ailleurs, un défaut d'oxydation mitochondriale des AG a également été proposé comme pouvant jouer un rôle causal dans l'accumulation d'espèces lipotoxiques (Lowell and Shulman, 2005). De nombreuses études ont en effet décrit une diminution de l'activité d'enzymes mitochondriales impliquées dans l'oxydation des lipides (Blaak *et al.*, 2000; Colberg *et al.*, 1995; He *et al.*, 2001; Simoneau *et al.*, 1995; Simoneau and Kelley, 1997; Simoneau *et al.*, 1999), associée à une diminution du nombre, de la taille et de l'activité des mitochondries avec l'obésité et le diabète de type 2 (Bajpeyi *et al.*, 2011; Kelley *et al.*, 2002; Morino *et al.*, 2005; Petersen *et al.*, 2004). Ces effets ont été en grande partie attribués à une diminution de l'expression de gènes impliqués dans la biogenèse mitochondriale tels que le *peroxysome proliferator-activated receptor γ coactivator 1 α* (PGC1α) (Mootha *et al.*, 2003). Ces résultats restent cependant purement associatifs, et l'hypothèse que les dysfonctions mitochondriales puissent être à l'origine de l'accumulation d'espèces lipotoxiques a été très débattue. Il a en effet été démontré qu'une invalidation spécifique de PGC1α dans le muscle squelettique entraîne une diminution du nombre de mitochondries qui est, de façon surprenante, associée à une amélioration de la sensibilité à l'insuline musculaire (Finck and Kelly, 2006; Handschin *et al.*,

2007). De plus, certaines études ont montré une diminution (Goodpaster, 2013; Kelley *et al.*, 1999) alors que d'autres ont montré une augmentation (Golay *et al.*, 1986; Holloszy, 2013; Holloway *et al.*, 2009) de l'oxydation lipidique dans le muscle de sujets obèses/diabétiques. Bien qu'une association entre dysfonction mitochondriale et insulino-résistance semble exister, il paraît difficile de déterminer si cette dysfonction est la cause ou l'origine de l'altération de la sensibilité à l'insuline musculaire. Il est toutefois important de noter qu'une surcharge lipidique mitochondriale peut entraîner une accumulation de dérivés d'oxydation incomplète des AG, notamment les acylcarnitines, dont la concentration musculaire est augmentée avec l'obésité (Aguer *et al.*, 2015; An *et al.*, 2004; Baker *et al.*, 2015; Koves *et al.*, 2005). Ces derniers peuvent altérer la signalisation insulinique, potentiellement via l'activation de PKC, ou d'autres protéines kinases induites par l'inflammation, entraînant l'inhibition d'IRS-1 (Koves *et al.*, 2008; McCoin *et al.*, 2015; Muoio and Newgard, 2008). Ces observations pourraient ainsi, indépendamment de l'accumulation d'espèces lipidiques lipotoxiques, constituer un lien entre dysfonction mitochondriale et développement de l'insulino-résistance.

Enfin, un lien entre la dérégulation de la lipolyse musculaire et l'accumulation d'espèces lipotoxiques telles que les DAG et les céramides a été récemment démontré par des travaux de notre laboratoire (Badin *et al.*, 2011) et va être davantage développé dans la suite de ce manuscrit.

6. DEREGULATION DE LA LIPOLYSE MUSCULAIRE ET INSULINO-RESISTANCE

La lipolyse musculaire, principalement régulée par le système nerveux sympathique, les hormones et la contraction musculaire (Watt and Spriet, 2004), consiste en l'hydrolyse des lMTG contenus dans les gouttelettes lipidiques en glycérol et acides gras (figure 14). Ces derniers peuvent être oxydés dans les mitochondries et constituent une source importante d'énergie pour la cellule musculaire, notamment à jeun ainsi que lors de la pratique d'un exercice physique d'intensité modérée. Cependant, il a été démontré qu'une dérégulation de la lipolyse musculaire, observée chez des sujets obèses/diabétiques, entraîne une accumulation de lipides intermédiaires lipotoxiques et joue ainsi un rôle causal dans le développement de l'insulino-résistance (Badin *et al.*, 2011; Badin *et al.*, 2013; Jocken *et al.*, 2010; Jocken *et al.*, 2008a). Il a été décrit que l'expression et l'activité des lipases musculaires sont dérégulées chez des sujets obèses et diabétiques, avec notamment une augmentation de *l'adipose triglyceride lipase* (ATGL) et une diminution de la lipase hormono-sensible (LHS) pouvant potentiellement conduire à une accumulation de DAG et de céramides chez ces individus (Badin *et al.*, 2011; Jocken *et al.*, 2010). Déterminer la façon dont la lipolyse musculaire est régulée permettra donc de mieux comprendre les mécanismes impliqués dans le développement de l'insulino-résistance et du diabète de type 2.

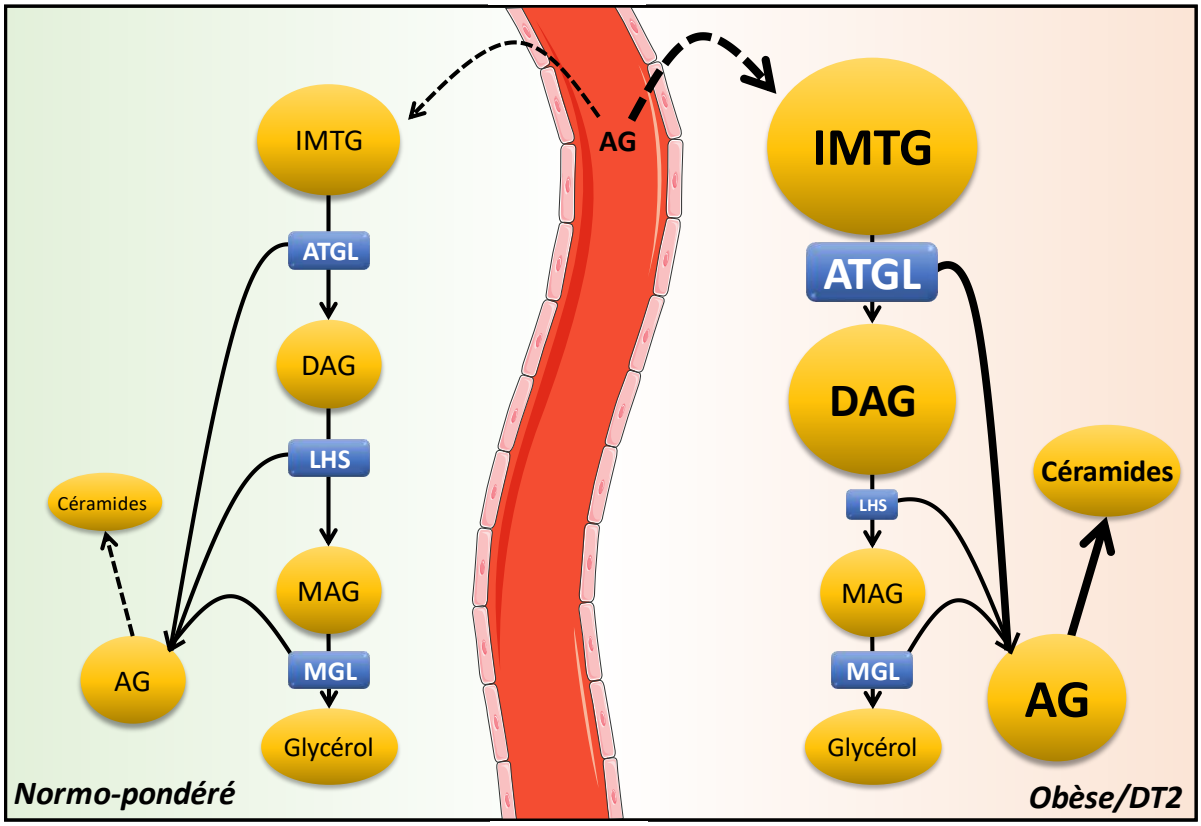

Figure 14. Dérégulation de la lipolyse et accumulation d'intermédiaires lipotoxiques

Chez les sujets obèses ou diabétiques de type 2 (DT2), l'activité de l'*adipose triglyceride lipase* (ATGL) est augmentée et celle de la lipase hormono-sensible (LHS) est diminuée. Ceci conduit à une accumulation de diacylglycérols (DAG) et d'acides gras (AG), ces derniers pouvant participer à la synthèse de céramides. MGL : monoacylglycérol lipase, IMTG : triacylglycérols intramyocellulaires

6.1. MONOACYLGLYCEROL LIPASE ET LIPASE HORMONO-SENSIBLE

La monoacylglycérol lipase (MGL) a été découverte en 1976 dans le tissu adipeux chez le rat, et a été décrite comme capable d'hydrolyser spécifiquement les monoacylglycérols (MAG) (Karlsson *et al.*, 1997; Tornqvist and Belfrage, 1976). De plus, une délétion de la MGL chez la souris entraîne une augmentation du contenu en MAG associée à une diminution de la libération d'AG et de glycérol par le tissu adipeux (Taschler *et al.*, 2011). De façon intéressante, ces souris sont également protégées de l'insulino-résistance induite par un régime gras, et son inhibition chez

des souris diabétiques améliore leur tolérance au glucose (Taschler *et al.*, 2011; Zhao *et al.*, 2014). Cependant, le rôle physiologique de la MGL dans la régulation de la lipolyse au sein du muscle squelettique n'a à ce jour pas été étudié.

La LHS a été identifiée en 1964 dans le tissu adipeux, et son activité a été décrite comme pouvant être régulée par les catécholamines (Khoo *et al.*, 1974; Vaughan *et al.*, 1964). La LHS est également exprimée dans le muscle squelettique et peut y être activée non seulement par les catécholamines mais également par la contraction musculaire (Holm *et al.*, 1987; Langfort *et al.*, 2003; Langfort *et al.*, 1998). Des études d'imagerie confocale ont notamment montré que la LHS cytosolique est relocalisée à la gouttelette lipidique en réponse à une stimulation par l'adrénaline ou lors de la contraction musculaire (Prats *et al.*, 2006). Ces observations suggèrent ainsi un lien entre localisation subcellulaire et activité de la LHS. Il a été décrit que l'adrénaline, via l'activation du récepteur β2-adrénergique, stimule la protéine kinase AMPc-dépendante (PKA), qui va à son tour phosphoryler la LHS sur des résidus activateurs (ser649 et ser650), entraînant ainsi une augmentation de son activité lipolytique (Krintel *et al.*, 2008; Talanian *et al.*, 2006). De façon intéressante, une diminution de la phosphorylation activatrice de la LHS a été décrite chez des individus obèses par rapport à des sujets normo-pondérés (Badin *et al.*, 2011). Par ailleurs, l'adrénaline joue également un rôle important dans l'activation de la LHS induite par l'exercice. Il a en effet été observé que l'augmentation de l'activité de la LHS induite lors de la pratique d'un exercice physique est fortement réduite chez des sujets adrénalectomisés (Kjaer *et al.*, 2000). La contraction musculaire semble toutefois être également capable d'activer la LHS de façon indépendante de l'adrénaline, notamment via l'activation de PKC et de ERK (*extracellular signal-regulated kinases*) (Donsmark *et al.*, 2003; Watt *et al.*, 2003a; Watt *et al.*, 2003b), probablement suite à une augmentation intracellulaire de la concentration de calcium (Langfort *et al.*, 2003).

Il a été montré que la LHS est capable d'hydrolyser les TAG et les DAG, mais présente une affinité dix fois plus importante pour les DAG que pour les TAG (Lass *et al.*, 2011). Par ailleurs, il a été observé *in vivo* chez les souris présentant une délétion de la LHS une accumulation de DAG, mais pas de TAG, au sein du tissu adipeux et du muscle squelettique (Haemmerle *et al.*, 2002), associée à une légère diminution de la sensibilité à l'insuline (Mulder *et al.*, 2003). Cet effet délétère sur l'insulino-

sensibilité musculaire est toutefois très modéré, malgré l'accumulation de DAG, probablement grâce à l'effet bénéfique de la diminution de la lipolyse adipocytaire sur la sensibilité à l'insuline (Girousse and Langin, 2012; Girousse *et al.*, 2013). Ces effets pourraient être en partie expliqués par une diminution des niveaux d'AG plasmatiques induite par l'inhibition de la lipolyse du tissu adipeux, protégeant ainsi le muscle d'une accumulation excessive de lipides ectopiques. Par ailleurs, il est également possible qu'une diminution de la lipolyse adipocytaire entraîne une réduction de la sécrétion d'adipokines connues pour jouer un rôle délétère dans la sensibilité à l'insuline systémique (Cao *et al.*, 2013; Ertunc *et al.*, 2015). En accord avec ces résultats, il a été montré que des souris dans lesquelles la LHS a été invalidée sont protégées du développement d'une insulino-résistance musculaire induite par un régime riche en graisses (Park *et al.*, 2005).

De façon intéressante, l'observation que les souris présentant une invalidation de la LHS ne présentent qu'une inhibition partielle de l'hydrolyse des TAG dans des adipocytes en culture (Okazaki *et al.*, 2002) et n'accumulent pas d'IMTG dans le muscle squelettique (Mulder *et al.*, 2003) laisse supposer qu'une autre enzyme puisse exister, responsable spécifiquement de leur hydrolyse.

6.2. ADIPOSE TRIGLYCERIDE LIPASE

L'*adipose triglyceride lipase* (ATGL) a été identifiée en 2004 dans le tissu adipeux (Jenkins *et al.*, 2004; Villena *et al.*, 2004; Zimmermann *et al.*, 2004), et a depuis été décrite dans de nombreux autres organes, dont le muscle squelettique (Jocken *et al.*, 2008b). La surexpression de l'ATGL dans des cultures d'adipocytes (Bezaire *et al.*, 2009) ou de cellules musculaires (Badin *et al.*, 2011) humaines induit une augmentation de la lipolyse des TAG. De plus, l'invalidation de l'ATGL chez la souris entraîne une forte prise de poids ainsi qu'une accumulation de TAG dans de nombreux organes, dont le tissu adipeux et le muscle squelettique (Haemmerle *et al.*, 2006). De façon surprenante, ces souris ne présentent pas d'altération de la sensibilité à l'insuline ni de la tolérance au glucose en régime normal (Haemmerle *et al.*, 2006), et sont protégées du développement d'une insulino-résistance induite par un régime riche en graisses (Hoy *et al.*, 2011). De façon marquante, l'activité de l'ATGL est corrélée négativement à la sensibilité à l'insuline chez l'homme, mesurée

lors d'un clamp hyperinsulinémique euglycémique, et sa surexpression dans des cultures primaires de cellules musculaires humaines entraîne une diminution de la signalisation et de l'action de l'insuline. Il est important de souligner que ces effets sont abolis lorsque la LHS est surexprimée de façon concomitante dans ces cellules, reflétant ainsi un rôle important de la balance entre l'activité de l'ATGL et de la LHS dans le maintien de la sensibilité à l'insuline (Badin *et al.*, 2011).

De façon intéressante, alors qu'il a été montré que les souris déficientes pour la LHS ne présentent pas de diminution de la lipolyse musculaire induite par la contraction (Alsted *et al.*, 2013), une augmentation du quotient respiratoire à l'exercice a été observée chez les souris déficientes pour l'ATGL, reflétant une utilisation plus importante des substrats glucidiques, associée à une diminution des stocks de glycogène musculaire (Huijsman *et al.*, 2009; Schoiswohl *et al.*, 2010). L'ensemble de ces travaux semble indiquer que l'ATGL est activée lors d'un exercice physique et favorise l'utilisation des substrats lipidiques au sein des cellules musculaires dans ce contexte.

De façon similaire à ce qui a été décrit pour la LHS, l'ATGL semble pouvoir être régulée par phosphorylation sur des résidus activateurs (ser404 et ser428) (Bartz *et al.*, 2007; Lass *et al.*, 2011) (figure 9). Il a en effet été démontré que la protéine kinase activée par l'AMP (AMPK) peut phosphoryler l'ATGL sur son résidu ser404, entraînant ainsi une augmentation de l'activité TAG hydrolase dans des adipocytes murins (Ahmadian *et al.*, 2011), et que la PKA est capable de phosphoryler l'ATGL sur ce même résidu dans les adipocytes (Pagnon *et al.*, 2012) et les cellules musculaires chez l'homme (Mason *et al.*, 2012). Une étude récente réalisée par l'équipe de H.S. Sul a démontré que les souris chez lesquelles l'AMPK a été invalidée spécifiquement dans le tissu adipeux présentent une diminution de la phorphorylation de l'ATGL sur son résidu ser404, associée à une réduction de la lipolyse basale (Kim *et al.*, 2016). Une augmentation de la phosphorylation de l'ATGL en ser404 a été décrite à jeun ainsi que lors de la pratique d'un exercice physique d'intensité modérée dans le tissu adipeux chez la souris, mais également en réponse à une stimulation β-adrénergique dans des explants de tissu adipeux sous-cutané humain (Pagnon *et al.*, 2012). Cependant, aucune augmentation de la phosphorylation du résidu ser404 n'a été observée dans le muscle squelettique humain suite à la pratique d'un exercice physique (Mason *et al.*, 2012). Identifier les

kinases responsables de la phosphorylation de l'ATGL sur son résidu ser428 pourrait permettre de mieux comprendre l'implication de la régulation par phosphorylation de cette enzyme dans le muscle, au repos et à l'exercice.

Indépendamment de sa capacité à être phosphorylée, l'ATGL est finement régulée par son association avec d'autres protéines, ayant un rôle activateur ou inhibiteur sur son activité lipolytique (figure 15). L'ATGL est positivement régulée par son co-activateur, la protéine *Comparative Gene Identification-58* (CGI-58). En 2006, A. Lass *et al.* ont démontré que CGI-58 interagit avec l'ATGL, et que cette interaction entraîne une augmentation de son activité TAG hydrolase (Lass *et al.*, 2006). Il a également été mis en évidence qu'une mutation de CGI-58 était responsable du syndrome de Chanarin Dorfman, entraînant une accumulation de lipides dans de nombreux organes, dont le muscle squelettique (Lass *et al.*, 2006; Lefevre *et al.*, 2001). La surexpression de CGI-58 dans des cultures primaires de cellules musculaires humaines entraîne une augmentation de l'activité de l'ATGL, une réduction de la quantité d'lMTG ainsi qu'une une augmentation de la lipolyse et de l'oxydation des AG (Badin *et al.*, 2012). Il a été décrit que CGI-58 est capable d'interagir avec la périlipine 1 dans les adipocytes, empêchant ainsi son interaction avec l'ATGL en condition basale (Subramanian *et al.*, 2004; Yamaguchi *et al.*, 2004). Lors d'une stimulation β-adrénergique, les phosphorylations de PLIN1 (Granneman *et al.*, 2009) et de CGI-58 (Sahu-Osen *et al.*, 2015) médiées par la PKA entraînent la libération de CGI-58, qui va alors pouvoir se lier à l'ATGL et augmenter son activité lipolytique. De façon intéressante, une augmentation de l'interaction entre l'ATGL et CGI-58 lors de la contraction musculaire a été décrite chez la souris (MacPherson *et al.*, 2013). Ceci suggère que CGI-58 pourrait également jouer un rôle important dans la régulation de la lipolyse musculaire induite par l'exercice physique, bien que les mécanismes moléculaires sous-jacents n'aient pas encore été clairement identifiés. De façon intéressante, une augmentation de l'expression de GCI-58 dans le muscle squelettique a été décrite chez des animaux nourris avec un régime gras (Badin *et al.*, 2013), ce qui pourrait contribuer à expliquer l'augmentation de l'activité de l'ATGL musculaire observée avec l'obésité. Cependant, aucune modification de l'expression de CGI-58 n'a été reportée dans le muscle de sujets obèses par rapport à des sujets normo-pondérés (Jocken *et al.*, 2010). Il est possible d'imaginer que, alors que le contenu total de CGI-58 est inchangé, une interaction plus importante avec l'ATGL

ait lieu chez l'homme dans le muscle de sujets obèses, expliquant les résultats à priori contradictoires décrits ci-dessus, et de futurs travaux de recherche seront nécessaires pour évaluer cette hypothèse.

Par ailleurs, il a récemment été décrit que l'activité de l'ATGL peut être inhibée par la protéine G_0/G_1 Switch Gene 2 (G0S2) dans le tissu adipeux (Schweiger *et al.*, 2012; Yang *et al.*, 2010) et dans le foie (Wang *et al.*, 2013; Zhang *et al.*, 2014). La surexpression de G0S2 dans des adipocytes isolés, ou dans des explants de tissu adipeux, entraîne une diminution de la lipolyse basale et stimulée. A l'inverse, une invalidation de G0S2 entraîne une augmentation de la lipolyse dans des adipocytes matures (Schweiger *et al.*, 2012; Yang *et al.*, 2010). Il a été montré que le domaine hydrophobe de G0S2 est capable d'interagir avec le domaine patatin-like de l'ATGL, et que cette interaction est nécessaire à l'inhibition de l'activité lipolytique de l'ATGL (Cornaciu *et al.*, 2011; Lu *et al.*, 2010). Le rôle fonctionnel de G0S2 a récemment été confirmé *in vivo* à l'aide de modèles animaux. Ainsi, les souris surexprimant G0S2 dans le tissu adipeux présentent une augmentation de la quantité de masse grasse associée à une diminution de la lipolyse adipocytaire et une réduction des taux de TAG circulants (Heckmann *et al.*, 2014). A l'inverse, des souris dont le gène codant pour G0S2 est délété prennent moins de poids lorsqu'elles sont nourries avec régime gras, ont une augmentation de la lipolyse adipocytaire, une diminution de la stéatose hépatique et sont protégées du développement d'une insulino-résistance (El-Assaad *et al.*, 2015).

De façon intéressante, il a été montré que G0S2 est également exprimé dans le muscle squelettique chez l'homme (Louche *et al.*, 2013). Nous avons émis l'hypothèse que G0S2 soit un inhibiteur de l'ATGL dans cet organe et joue un rôle important dans le contrôle du métabolisme énergétique musculaire, c'est ce qui a fait l'objet du deuxième article présenté dans ce manuscrit.

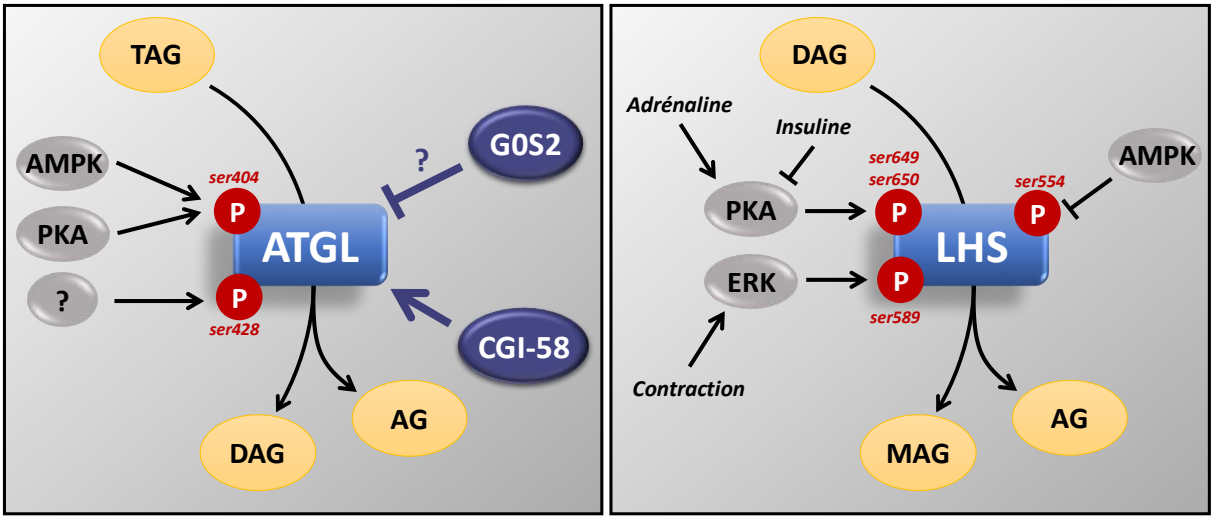

Figure 15. Régulation de l'adipose triglycéride lipase et de la lipase hormono-sensible

L'adipose triglycéride lipase (ATGL) est régulée par phosphorylation par la protéine kinase activée par l'AMP (AMPK) et par la protéine kinase A (PKA). Elle est également co-activée par le *comparative gene identification-58* (CGI-58) et potentiellement inhibée par le G_0/G_1 *switch gene 2* (G0S2). La lipase hormono-sensible (LHS) est régulée par phosphorylation par la PKA, par l'AMPK et par *extracellular signal-regulated kinases* (ERK). TAG : triacylglycerol ; DAG : diacylglycerol ; AG : acide gras ;

6.3. PERILIPINES

Les gouttelettes lipidiques, dans lesquelles sont stockés les IMTG, sont composées d'un cœur de lipides neutres entouré d'une couche de phospholipides à leur surface (Fujimoto and Ohsaki, 2006; Olofsson *et al.*, 2009; Tauchi-Sato *et al.*, 2002) dans laquelle sont insérées de nombreuses protéines, les plus représentées appartenant à la famille des périlipines. Une importante hétérogénéité de ces gouttelettes a été décrite, notamment au niveau de leur taille, leur localisation subcellulaire et tissulaire ainsi que dans la composition des protéines de surface qui les recouvrent (Bosma, 2016; Ducharme and Bickel, 2008). Cinq isoformes de périlipines ont été identifiées jusqu'à présent, et présentent des spécificités d'expressions tissulaires décrites dans le tableau 1 (Bickel *et al.*, 2009; Sztalryd and Kimmel, 2014).

PLIN1 (Perilipin, PERI)	Tissu adipeux blanc, tissu adipeux brun
PLIN2 (Adipophilin, ADRP, ADFP)	Ubiquitaire
PLIN3 (TIP47, PP17, M6PRBP1)	Ubiquitaire
PLIN4 (S3-12)	Ubiquitaire
PLIN5 (PAT-1, OXPAT, LSDP5, MLDP)	Coeur, Muscle squelettique, Foie, Tissu adipeux brun

Tableau 1. Expression tissulaire des différentes isoformes de périlipines
Les différents isoformes de périlipines présentent des différences d'expression tissulaire. La périlipine 1 (PLIN1) est notamment exprimée dans les tissus adipeux, et la périlipine 5 (PLIN5) dans les tissus oxydatifs. Les autres isoformes de périlipine ont une expression ubiquitaire. ADRP, ADFP : *Adipose Differenciation-Related Protein* ; TIP47 : *Tail-Interacting Protein, 47kDa* ; PP17 : *Placental Protein 17* ; M6PRBP1 : *Mannose-6-Phosphate Receptor Binding Protein 1* ; S3-12 : *Adipocyte Protein S3-12 ;* PAT-1 : *Perilipin ADRP TIP47* -1 ; OXPAT : *Oxidative tissues-enriched PAT protein* ; LSDP5 : *Lipid Storage Droplet Protein 5* ; MLDP : *Myocardial Lipid Droplet Protein*

Il a été montré que ces protéines jouent un rôle important dans la dynamique des gouttelettes lipidiques. En condition basale, les périlipines protègent les gouttelettes lipidiques de leur hydrolyse par les lipases présentes dans le cytosol (*i.e.* ATGL, LHS et MGL). En revanche, lors d'une stimulation de la lipolyse, par exemple par les catécholamines, les périlipines sont capables de recruter les lipases à la surface des gouttelettes lipidiques afin de potentialiser leur action lipolytique (Bickel *et al.*, 2009).

Les périlipines 2, 3, 4 et 5 sont toutes les quatre exprimées dans le muscle squelettique (Gjelstad *et al.*, 2012). La périlipine 5 (PLIN5) a la particularité d'être fortement exprimée dans les tissus oxydatifs comme le cœur, le foie, le tissu adipeux brun ainsi que le muscle squelettique (Dalen *et al.*, 2007; Wolins *et al.*, 2006b), ce qui suggère qu'elle puisse jouer un rôle particulier dans le métabolisme oxydatif. Il a été montré que PLIN5 joue un rôle de « barrière lipolytique » dans différents organes, protégeant ainsi les gouttelettes lipidiques de leur dégradation par les lipases (Pollak

et al., 2013; Wang *et al.*, 2015). De façon intéressante, une surexpression de PLIN5 dans le muscle squelettique chez la souris protège la gouttelette lipidique et entraîne une accumulation d'IMTG (Bosma *et al.*, 2013). Par ailleurs, différentes études ont permis de mettre en évidence que PLIN5 est capable d'interagir avec l'ATGL et CGI-58 au repos, et uniquement avec l'ATGL en condition de lipolyse stimulée (Granneman *et al.*, 2011; MacPherson *et al.*, 2013; Mason and Watt, 2015). Il a ainsi été suggéré que, en condition basale, PLIN5 séquestrerait l'ATGL d'une part, et CGI-58 d'autre part. En revanche, en condition de lipolyse stimulée, PLIN5 libèrerait CGI-58, lui permettant de se lier à l'ATGL et d'augmenter son activité d'hydrolyse des TAG. Ces résultats sont en accord avec une étude de l'équipe de C.S. Shaw qui a montré que suite à la pratique d'un exercice physique, les IMTG contenus dans les gouttelettes recouvertes par PLIN5 sont préférentiellement hydrolysés (Shepherd *et al.*, 2013), suggérant ainsi que PLIN5 favoriserait l'utilisation des réserves lipidiques à l'exercice. Un rôle facilitateur direct de PLIN5 sur l'oxydation des AG en période de forte demande énergétique n'a toutefois pas été à l'heure actuelle démontré dans le muscle squelettique. De plus, il a été montré que la surexpression musculaire de PLIN5 chez la souris induit l'augmentation d'un cluster de gènes impliqués dans le contrôle du catabolisme des AG et de l'oxydation mitochondriale (Bosma *et al.*, 2013). De façon surprenante, des études de différents groupes de recherche ont mis en évidence que PLIN5 serait également présente à la surface des mitochondries (Bosma *et al.*, 2012; Wang *et al.*, 2011; Wang and Sztalryd, 2011). Par ailleurs, il a également été démontré que PLIN5 peut être phosphorylée par la PKA suite à une stimulation β-adrénergique (Pollak *et al.*, 2015; Wang *et al.*, 2011). L'ensemble de ces résultats suggère que, de la même façon que ce qui a été précédemment décrit pour PLIN1 dans le tissu adipeux, PLIN5 serait capable de protéger la gouttelette lipidique au repos, et de favoriser la lipolyse et l'adressage des AG à la mitochondrie lorsque les besoins énergétiques de la cellule augmentent.

Cependant, le rôle fonctionnel de PLIN5 n'a à l'heure actuelle pas été étudié dans le muscle squelettique humain, et c'est ce qui a fait l'objet de la troisième publication présentée dans ce manuscrit.

7. OBJECTIFS DE THESE

Il est maintenant communément admis que deux dépôts lipidiques distincts (*i.e.* IMAT et IMTG) sont présents au sein du muscle squelettique, et que, lorsqu'ils sont présents en excès, ces lipides sont associés à la mise en place de l'insulino-résistance musculaire chez l'homme. Cependant, le rôle causal de ces lipides dans l'altération de la sensibilité à l'insuline, ainsi que les mécanismes moléculaires impliqués, ne sont pas clairement établis.

Le but de ma thèse a par conséquent été de contribuer à :

1. La caractérisation fonctionnelle et phénotypique des progéniteurs adipocytaires présents dans le muscle squelettique chez l'homme, ainsi que la mise en évidence du rôle causal des adipocytes qui en dérivent dans le développement de l'insulino-résistance musculaire.

2. L'amélioration des connaissances portant sur le contrôle de la lipolyse musculaire chez l'homme, notamment via l'étude du rôle fonctionnel de G0S2 et PLIN5, deux protéines impliquées dans régulation de la dynamique des gouttelettes lipidiques, ainsi que leur lien mécanistique et causal avec l'insulino-résistance musculaire chez la souris.

8. RESULTATS

8.1. PUBLICATION 1 : LES PROGENITEURS ADIPOCYTAIRES ISOLES DU MUSCLE SQUELETTIQUE DE SUJETS OBESES HUMAINS DONNENT NAISSANCE A DES ADIPOCYTES BLANCS FONCTIONNELS QUI CONTRIBUENT AU DEVELOPPEMENT DE L'INSULINO-RESISTANCE

Plusieurs études chez l'homme ont décrit une corrélation positive entre la quantité d'IMAT et (i) l'IMC, (ii) l'insulino-résistance et (iii) le diabète de type 2 (Boettcher *et al.*, 2009; Gallagher *et al.*, 2009; Hilton *et al.*, 2008; Marcus *et al.*, 2010). Nous avons émis l'hypothèse que la proximité entre ces adipocytes et les fibres musculaires pourrait, de façon paracrine, jouer un rôle dans le développement de l'insulino-résistance.

Nous avons donc cherché dans un premier temps à isoler les progéniteurs donnant naissance à ces adipocytes, à partir de cultures primaires de fraction stroma-vasculaire (SVF) issues de biopsies de muscle squelettique provenant de sujets obèses, par une approche de tri cellulaire par cytométrie de flux sur la base des marqueurs de surface exprimés par les cellules satellites (*i.e.* progéniteurs musculaires) et les FAP (*i.e.* progéniteurs adipocytaires). Nous avons ensuite mis en culture les FAP ainsi isolés, évalué leur potentiel de différentiation adipogénique et caractérisé leurs propriétés métaboliques. Enfin, nous avons, par des expériences de milieux conditionnés, cherché à établir l'existence d'un dialogue entre FAP différenciés en adipocytes et fibres musculaires, et évalué son impact sur la sensibilité à l'insuline de ces dernières.

8.1.1. Publication 1

Adipogenic progenitors from obese human skeletal muscle give rise to functional white adipocytes that contribute to insulin resistance

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ORIGINAL ARTICLE

Adipogenic progenitors from obese human skeletal muscle give rise to functional white adipocytes that contribute to insulin resistance

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BACKGROUND/OBJECTIVES: Recent reports indicate that inter/intramuscular adipose tissue (IMAT), composed by adipocytes underneath the deep fascia of the muscles, is positively correlated with aging, obesity and insulin resistance in humans. However, no molecular/cellular evidence is available to support these interactions. The current study aimed to better characterize human skeletal muscle-derived adipogenic progenitors obtained from obese volunteers and investigate the impact of derived adipocytes on insulin action in primary skeletal muscle cells.

METHODS: Primary cultured stroma-vascular fraction (SVF) obtained from *vastus lateralis* muscle biopsies of middle-aged obese subjects was immunoseparated (magnetic beads or flow cytometry). The characteristics and/or metabolic phenotype of $CD56^+$, $CD56^-$ and $CD56^-CD15^+$ cellular fractions were investigated by complementary approaches (flow cytometry, cytology, quantitative PCR and metabolic assays). The effects of conditioned media from $CD56^-CD15^+$ cells differentiated into adipocytes on insulin action and signaling in human primary myotubes was also examined.

RESULTS: Our data indicate that $CD56^+$ and $CD56^-$ cellular fractions isolated from cultured SVF of human muscle contain two distinct committed progenitors: $CD56^+$ cells (that is, satellite cells) as myogenic progenitors and $CD15^+$ cells as adipogenic progenitors, respectively. $CD56^-CD15^+$-derived adipocytes display the phenotype and metabolic properties of white adipocytes. Secretions of $CD56^-CD15^+$ cells differentiated into functional white adipocytes reduced insulin-mediated non-oxidative glucose disposal ($P = 0.0002$) and insulin signaling.

CONCLUSIONS: Using *in-vitro* models, we show for the first time that secretions of skeletal muscle adipocytes are able to impair insulin action and signaling of muscle fibers. This paracrine effect could explain, at least in part, the negative association between high levels of IMAT and insulin sensitivity in obesity and aging.

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INTRODUCTION

Inter/intramuscular adipose tissue (IMAT) is an ectopic fat depot underneath the deep fascia of the muscles and is measurable via computed tomography and magnetic resonance imaging. IMAT includes adipocytes located between muscle fibers and between muscle groups. High level of IMAT was found in skeletal muscles of dystrophic, obese, diabetic, sedentary and elderly individuals.[1–3] This excess storage of lipids in skeletal muscle adipocytes was shown to be positively correlated with adiposity, systemic insulin resistance, loss of strength and reduced mobility.[3–5] Interestingly, after adjusting for body mass index, IMAT remained a strong predictor of fasting blood glucose and insulin levels, suggesting that metabolic impairments are not simply due to obesity alone.[6] We thus hypothesized that the vicinity of these adipocytes to myofibers may contribute to metabolic dysfunction and insulin resistance in a paracrine manner.

The origin of skeletal muscle adipocyte progenitors is still a matter of debate. Indeed, although skeletal myogenic progenitors (that is, satellite cells) naturally exhibit myogenic differentiation potential, they have been reported to express osteogenic and adipogenic differentiation abilities.[7] These alternative fates may represent multipotency, transdifferentiation or amplification of distinct cell subpopulations within the satellite cell niche.[8] However, recent literature using both human or mouse skeletal muscle-derived cells have established that two distinct mesenchymal progenitors contribute to myofibers and adipocytes in the skeletal muscle.[9,10] Whereas CD56 (NCAM, neural cell adhesion molecule) is well known to be a marker of human skeletal muscle satellite cells,[11] there is no consensus on the antigen expression pattern expressed by adipogenic progenitors. Among candidates, the carbohydrate adhesion molecule CD15 (3-fucosyl-*N*-acetyl-lactosamin) has been identified by different groups to be expressed at the cell surface of on human skeletal muscle-derived adipogenic progenitors.[9,12,13] However, in a recent article its expression was reported to be totally absent on human skeletal muscle-derived adipogenic progenitors, reviving the debate.[14] Of note, human skeletal muscle-derived adipogenic progenitors studied until now were obtained from healthy (without obesity or diabetes) lean subjects.

Hence, the goal of the current study was to better characterize the phenotype of human skeletal muscle-derived adipogenic progenitors/adipocytes obtained from obese male volunteers and

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to investigate their paracrine effect on insulin sensitivity of human primary myotubes. Here we show that $CD15^+$ cells isolated from the $CD56^-$ fraction of cultured human skeletal muscle-derived stroma of obese subjects display the features and metabolic phenotype of white adipocytes and, for the first time, that they have a negative paracrine impact on insulin action in human primary myotubes.

MATERIAL AND METHODS

Source of human muscle biopsies

Muscle biopsies of *vastus lateralis* weighing 60–100 mg each were obtained, using the Bergstrom technique, from middle-aged obese subjects (10 men, age: 38 ± 1.5 years, body mass index: 33 ± 0.7 kg m^{-2}) that were enrolled in a clinical trial (NCT01083329 and EudraCT-2009-012124-85) in Toulouse (France). In some experiments, myogenic progenitors of *rectus abdominis* biopsies from middle-aged, lean healthy subjects (4 men, age: 34 ± 2.5 years, body mass index: 26 ± 1.4 kg m^{-2}) were used, as previously published.[15,16] Both protocols were approved by the institutional review board and the local ethics committee. All volunteers gave written informed consent.

Muscle tissue was collected in Dulbecco's modified Eagles's medium (DMEM) low glucose-Glutamax (Life Technologies, Saint-Aubin, France)/penicillin–streptomycin 2%/fungizone 0.2% for primary cell culture.

Primary cell culture

Chemicals and culture media were from Sigma-Aldrich (Saint-Quentin Fallavier, France) and Life Technologies. Plates (6, 12 and 24 wells) and flask (T25 and 75) were provided from TPP (Dutscher, Issy-Les-Moulineaux, France).

Pieces of 40–60 mg of total muscle biopsies were digested in a trypsin 0.25%/collagenase type IV 0.068%/EDTA 0.05%/bovine serum albumin (BSA) 0.1% solution for 30 min (37 °C, mild agitation) to isolate the stroma vascular fraction (SVF). Cells from the SVF (~2000 cells per cm^2) were grown in T25 flask in proliferation medium 1 (Supplementary Table S1) to reach confluence. Cells were then trypsinized (0.05% trypsin-EDTA) and frozen (1×10^6 cells per cryotube) in a cryopreservation medium (DMEM, fetal bovine serum 16%, dimethyl sulfoxide 10%) till use.

Cell sorting

Thawed SVF cells obtained from obese volunteers were amplified in T75 flasks (12 500 cells per cm^2, passage 2) in proliferation medium 2 (Supplementary Table S1) until confluence. Quiescent and activated satellite cells (that is, myoblasts) were immunopurified by the use of the mouse monoclonal 5.1H11 anti-CD56 antibody (DSHB, Iowa City, IA, USA) and MACS cell separation column technology (Miltenyi Biotech SAS, Paris, France).[11] The composition of both SVF and the two sorted cell fractions (100 000 cells of each) were analyzed by flow cytometry (BD Biosciences FACSCanto flow cytometer and FACSDiva software, Le Pont de Claix, France) using PE-Cy7-CD56, APC-CD15, PerCP-D45 and FITC-CD31 mouse anti-human antibodies or isotype controls (BD Biosciences). For specific skeletal muscle adipocyte progenitor cell isolation (that is, $CD56^-CD15^+$ cellular fraction), fluorescence-activated cell sorting analysis was performed (BD Biosciences, BD Influx) using the panel of antibodies described above.

Cell culture

Following cell sorting, cells (passage 3) were plated in 24- or 12-well plates (12 500 cells per cm^2) and grown in proliferation medium 2 (Supplementary Table S1). Differentiation was initiated by switching to myogenic or adipogenic media (Supplementary Table S1). The medium was changed every 2 days and cells were grown typically up to 5 days (Day 0 to Day 4) for myogenic differentiation and 7 days (Day 0 to Day 6) for adipogenic differentiation.

According to the needs of the experiments, undifferentiated (D0) and/or differentiated cells were (i) lysed in RLT/dithiothreitol (2.5 mM) lysis buffer and stored at −20 °C for further RNA extraction, (ii) fixed in 4% paraformaldehyde and stored at 4 °C for cytochemical analysis and (iii) used for metabolic assays or triacylglycerol (TG) content evaluation.

To perform adipocyte-conditioned media, differentiated $CD56^-CD15^+$ cells (Day 6) were cultured 24 h in DMEM low glucose-Glutamax/fatty acid (FA)-free BSA 0.4%/penicillin–streptomycin 1%. Control medium were prepared using the same medium applied on empty wells for 24 h. Control and conditioned media were collected, centrifuged (14 000 r.p.m., 3 min) and frozen until use.

Determination of TG and protein contents

Protein contents were measured with Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA), according to the manufacturer's instructions, after cells were scrapped in various buffers.

For TG content evaluation, adipocytes resulting from differentiated $CD56^-$ and $CD56^-CD15^+$ cellular fractions (Day 6) were scrapped in phosphate-buffered saline (PBS)/Tween 0.2% and sonicated. Total glycerol (Free Glycerol Reagent, Sigma-Aldrich) and TG-derived glycerol (TG Reagent, Sigma-Aldrich) levels were then measured successively. TGs contained in adipocytes was evaluated by the subtraction of both absorbances.

Reverse-transcription and real-time quantitative PCR

Total RNA from cultured cells was isolated in Qiagen (Hilden, Germany) RNeasy mini kit according to the manufacturer's instructions. The quantity of the RNA was determined on a Nanodrop ND-1000 (Thermo Scientific). RNA was reverse transcribed using using the Multiscribe Reverse Transcriptase method on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR was performed to determine cDNA content. Primers and probes were bought from Applied Biosystems and Eurogentech (Seraing, Liège, Belgium; Supplementary Table S2). The amplification reaction was performed in duplicate on 10 ng of the cDNA in 96-well reaction plates on a StepOnePLus system (Applied Biosystems) as previously described.[17] All expression data were normalized by the $2^{(\Delta Ct)}$ method using 18S rRNA as the internal control.

Immunocytochemistry

Fixed cells were permeabilized for 20 min in PBS/0.5%Triton. After rinsing, cells were incubated in PBS/glycine (100 mM, 15 min), then in PBS/3% BSA (30 min) and finally in PBS/0.1% BSA/0.2% Triton/0.05% Tween containing mouse primary antibody directed against human sarcomeric myosin (MF20, DSHB, 1/4, overnight, 4 °C). Cells were rinsed and incubated with the corresponding fluorescence-labeled secondary antibody (goat anti-mouse-AlexaFluor 546, Invitrogen (Life Technologies), 1/250, 90 min). Cells were washed and incubated with 5 μg ml^{-1} Hoechst 33242 to stain nuclei and 2 μg ml^{-1} BODIPY 493/503 (Invitrogen, 15 min) to stain droplets containing neutral lipids and washed again before direct observation with a fluorescence microscope (Nikon TE2000, Champigny-Sur-Marne, France). Representative images were recorded (Image analysis system LUCIA).

Lipolysis assay

Adipocytes obtained from differentiated $CD56^-$ and $CD56^-CD15^+$ cellular fractions (Day 6) were pulsed overnight with [1-^{14}C]oleate (1 μCi ml^{-1}, PerkinElmer, Courtabeuf, France) and cold oleate (80 μM), to pre-label the endogenous TG pool. Following the pulse, cells were chased for 3 h, in the presence or absence of forskolin (10 μM) or atrial natriuretic peptide (1 μM), to stimulate lipolysis, in DMEM glucose 0.1 mM/FA-free BSA 0.25%/triacsin C 10 mM (a blocker of FA recycling into TG pools[15,18]). Adipocytes were collected in SDS 0.1% at the end of the chase period, to determine protein content, and FA release into the medium was counted by liquid scintillation. All assays were performed in triplicates and data were normalized to cell protein content.

Glycogen synthesis assay

Myogenic progenitors (passage 4) from lean healthy donors were grown and differentiated in 24-well plates as described in the cell culture section. At day 4 of differentiation, culture medium of myotubes was replaced by (i) control medium/DMEM, low glucose, Glutamax or (ii) adipocyte-conditioned medium/DMEM low glucose-Glutamax (v/v) for an overnight period (18 h).

Myotubes were then exposed to new DMEM, low glucose, Glutamax supplemented with D[U-^{14}C]glucose (1 μCi ml^{-1}, Perkinelmer) in the presence or absence of 100 nM of insulin for 3 h, to study basal and insulin-mediated glycogen synthesis, as previously described.[16] After rapidly rinsing each well twice with PBS, the cells were solubilized by the addition of KOH 30%. The samples were added of glycogen (Sigma-Aldrich) 60 mg ml^{-1} in distilled water and heated at 80 °C for 20 min.

Following incubation, ice-cold absolute ethanol was added to precipitate glycogen. The tubes were then centrifuged (10 000 r.p.m., 20 min, 4 °C) and the supernatant was immediately removed and discarded. After one wash with ethanol 70%, the glycogen precipitate was re-suspended in distilled water, dissolved under shaking for 20 min and counted by liquid scintillation. All assays were performed in triplicates and data were normalized to protein content.

Glucose uptake assay

$CD56^-$ cellular fraction differentiated in adipogenic conditions (D6) or primary myotubes from lean subjects (D4), pre-incubated with control or adipocyte-conditioned/DMEM, low glucose, Glutamax media (as described above), were serum starved for 2 h in DMEM without glucose. Cells were then incubated for 10 min in DMEM without glucose containing a mix of cold 2-deoxy-glucose (10 μM) and [1,2-^{3}H]2-deoxy-glucose (1 μCi ml^{-1}, PerkinElmer) in the absence or presence of 100 nM of insulin. Cells were rinsed, lysed in SDS 0.1% and sonicated. Radioactivity in cell lysates was counted in duplicate on a β-counter (PerkinElmer). All assays were performed in triplicates and data were normalized to protein content.

Western blot analysis

Myogenic progenitors (passage 4) from lean healthy donors were grown and differentiated in six-well plates as described in the cell culture section. At day 4 of differentiation, culture medium of myotubes was replaced by (i) control medium/DMEM, low glucose, Glutamax or (ii) adipocyte-conditioned medium/DMEM, low glucose, Glutamax (v/v) for an overnight period (18 h).

Myotubes were then exposed to α-Minimum Essential Medium Eagle, low glucose, Glutamax in the presence or absence of 100 nM of insulin for 20 min, to study basal and insulin activation pathway. They were then scrapped in RIPA buffer (Sigma-Aldrich) supplemented with 10 μl ml^{-1} protease and phosphatase I and II inhibitor (Sigma-Aldrich), and snap frozen. Proteins (25 μg) from myotubes were run on a 4–20% SDS-polyacrylamide gel electrophoresis (Bio-Rad, Marnes-La-Coquette, France), transferred onto nitrocellulose membranes (Hybond ECL, Amersham Biosciences, Velizy-Villacoublay, France) and washed with Tris-buffered saline Tween 0.2% (TBS-T)/5% milk (1 h, 4 °C). Membranes were then incubated with primary antibodies against total Akt and phospho-Akt (Ser473, Thr308) (1/1000 in TBS-T/milk, overnight, 4 °C), washed in TBS-T, then with anti-rabbit horseradish peroxidase-linked secondary antibodies (1/10 000 in TBS-T/milk, 1 h, 4 °C). Subsequently, and after washing in TBS-T, immunoreactive proteins were visualized using the ChemiDoc MP Imaging System and data analyzed using the Image Lab 4.1 version software (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (1/10 000) served as an internal control. Antibodies were from Cell Signaling Technology (Ozyme, St Quentin en Yveline, France).

Statistical analyses

Statistical analyses were performed using GraphPad Prism 5.0 for Windows (GraphPad Software Inc., La Jolla, CA, USA). Normal distribution and homogeneity of variance of the data were tested using Shapiro–Wilk and F tests, respectively. Student's *t*-tests or Mann–Whitney tests were performed to determine the effect of a treatment (for example, forskolin, atrial natriuretic peptide, insulin and so on) or the day of differentiation on one parameter. Two-way analyses of variance were applied to determine the effect of two variables (for example, day of differentiation and cellular fractions) on one parameter. Bonferroni's *post-hoc* tests were applied when interactions were found. All values in figures and table are presented as mean ± s.e.m. Statistical significance was set at $P < 0.05$.

RESULTS

$CD56^+$ and $CD56^-$ cellular fractions isolated from cultured skeletal muscle stroma contains two progenitor cells with distinct fates

CD56 plasma membrane marker is expressed on human progenitors of skeletal muscle fibers, the satellite cells.[11,19] Thus, $CD56^+$ cellular fraction has been extensively isolated, differentiated and used as primary culture of myotubes. In addition, recent articles have reported the existence of adipogenic progenitors, not expressing the CD56 marker, in skeletal muscle-derived SVF of lean individuals.[9,13] In a well-defined cohort of obese subjects, we studied the phenotype of isolated $CD56^+$ and $CD56^-$ cellular fractions cultured in myogenic or adipogenic conditions.

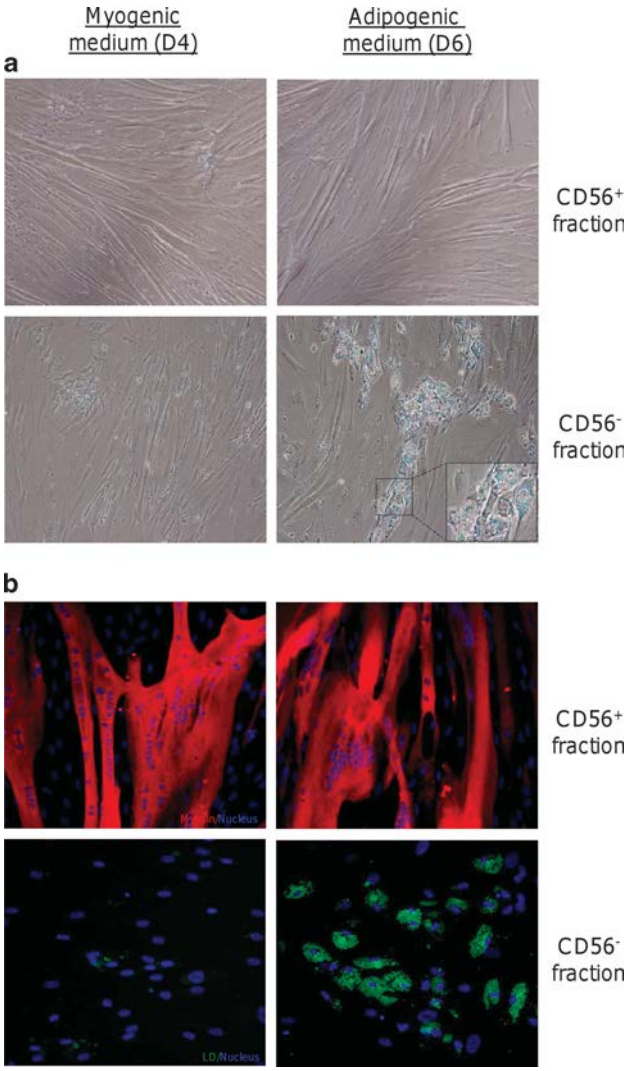

Figure 1. Microscopy analyses of both $CD56^+$ and $CD56^-$ cellular fractions cultured in myogenic and adipogenic conditions. (**a**) Representative picture of $CD56^+$ and $CD56^-$ cellular fractions cultured for 5 days in myogenic medium (D4) and for 7 days in adipogenic medium (D6) ($n = 6$). Original magnification, ×100. (**b**) Representative picture of nuclei (blue), sarcomeric human myosin (red) or neutral lipids (green) in $CD56^+$ and $CD56^-$ cellular fractions cultured for 5 days in myogenic medium (D4) and for 7 days in adipogenic medium (D6) ($n = 3$). Original magnification, ×200.

Immunomagnetic selection of $CD56^+$ and $CD56^-$ cellular fractions was successfully performed, as $CD56^+$ cells were present at almost 90 ± 4.2% in the $CD56^+$ fraction and nearly absent in the $CD56^-$ fraction (1.9 ± 0.7% of $CD56^+$ cells). Both isolated fractions were then cultured in myogenic (Day 0 to Day 4) and in adipogenic (Day 0 to Day 6) media. Cells from the $CD56^+$ fraction, when cultured in both media, fused with each other giving rise to multinucleated myofibers (Figures 1a and b). As expected, $CD56^+$ cells cultured in myogenic medium expressed sarcomeric myosin (Figure 1b) as well as classical genes involved in myogenic differentiation process (Figure 2a). In agreement with the literature, mRNA expression of *PAX7* and *MYF5* was decreased, whereas transcript levels of *MYOG*, *MYH7* (Figure 2a), *MHY2* and *MHY1* ($n = 5$, data not shown) were increased as differentiation occurs. Of note and in agreement with microscopic observation (Figures 1a and b), *MHY7* mRNA level and expression profile were

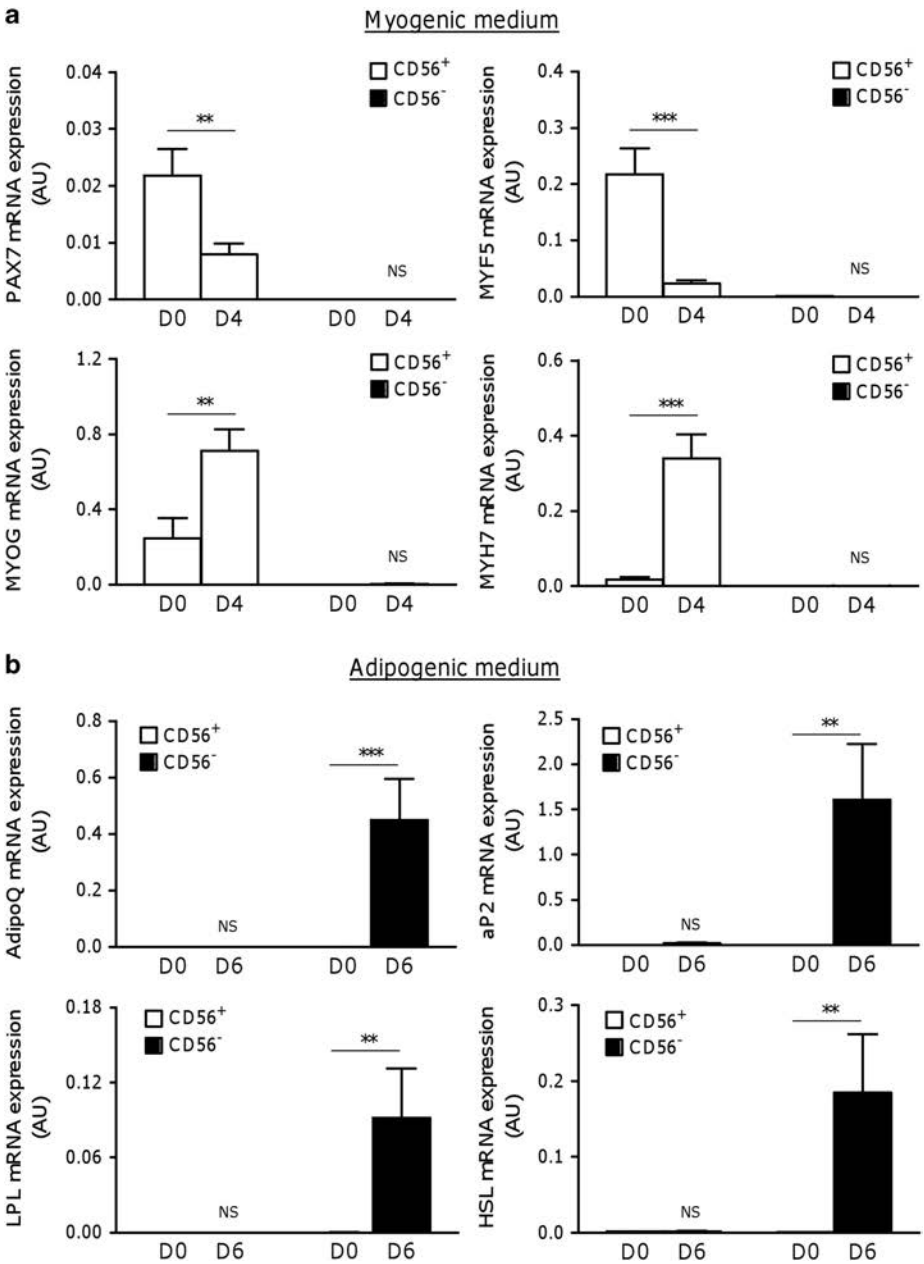

Figure 2. Gene expression analyses of both CD56$^+$ and CD56$^-$ cellular fractions cultured in myogenic and adipogenic conditions. CD56$^+$ and CD56$^-$ cellular fractions were cultured for 5 days in myogenic medium (D4) or for 7 days in adipogenic medium (D6), and mRNA expression of myogenic-related genes (**a**) and adipogenic-related genes (**b**), respectively, were assessed by quantitative PCR. Two-way analysis of variance revealed a significant effect of cellular fraction (CD56$^+$ vs CD56$^-$) and day of differentiation (D0 vs D4 or D0 vs D6) on mRNA expression, with interactions ($n = 4$–6). $^{**}P < 0.01$ and $^{***}P < 0.001$ D0 vs D4 or D6. NS, not significant.

similar in CD56$^+$ cells cultured in myogenic and adipogenic conditions ($n = 5$, data not shown), suggesting that these cells were already committed to the muscle lineage.

Interestingly, CD56$^-$ cellular fraction does not exhibit a characteristic phenotype in myogenic medium as shown by microscopic observations (Figures 1a and b). They express very low levels of myogenic transcripts (Figure 2a). In contrast, when cultured in adipogenic medium, these CD56$^-$ cells acquire, after 7 days of differentiation, an adipocyte-like phenotype characterized by round shape morphology, numerous refractive lipid droplets containing neutral lipids (Figures 1a and b) and the significant expression of adipocyte-related genes (*AdipoQ*, *aP2*, *LPL* and *HSL*) (Figure 2b). It has to be noted that in contrast, CD56$^+$ cellular fraction expressed very low amounts of these adipocyte-related genes (Figure 2b).

In conclusion, phenotypic analyses of both CD56$^+$ and CD56$^-$ cellular fractions isolated from human skeletal muscle-derived primary cultured SVF reveal the presence of two distinct committed progenitor cells.

CD56$^-$ cellular fraction and, more specifically, its main constituting cell type, the CD15$^+$ cells, exhibit numerous metabolic properties of white adipocytes

We next decided to further investigate the functional characteristic and the metabolic phenotype of CD56$^-$ cellular fraction.

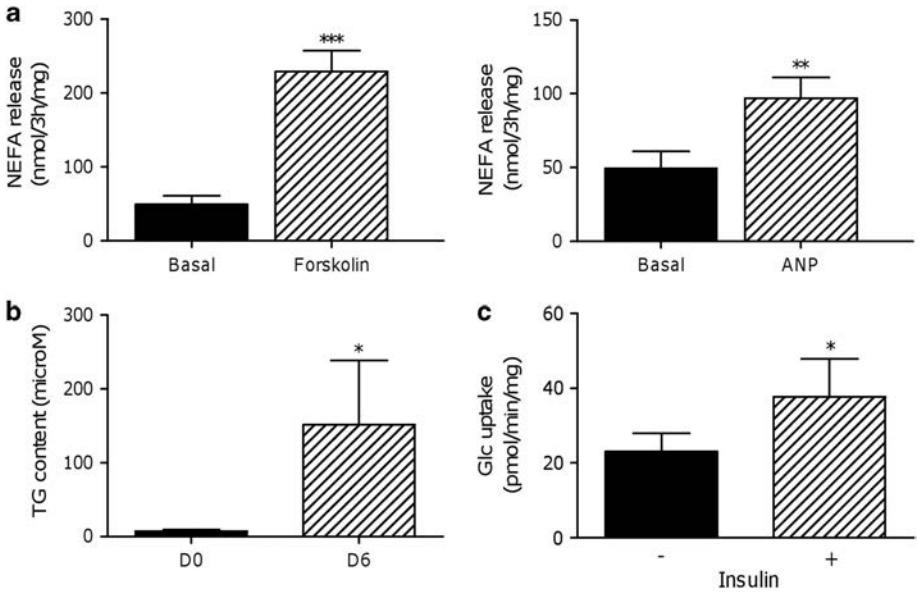

Figure 3. Functional analyses of CD56$^-$ cellular fraction cultured in adipogenic conditions. (**a**) Lipolysis capabilities of differentiated CD56$^-$ cellular fraction (D6) were analyzed by pulse chase using oleate to label TG pools and forskolin (10 μM) or atrial natriuretic peptide (ANP, 1 μM) to stimulate non-esterified fatty acid (NEFA) release from these pools ($n=6$). **$P<0.01$ and ***$P<0.001$ basal vs stimulated. (**b**) TG storage at the D0 and D6 of adipogenic differentiation ($n=5$). *$P<0.05$ D0 vs D6. (**c**) CD56$^-$ cellular fraction was cultured in adipogenic medium (D6), radiolabeled with D-deoxy-glucose and insulin (100 nM)-stimulated glucose uptake evaluated ($n=3$). *$P<0.05$ unstimulated vs stimulated.

Some reports have suggested the presence of a brown adipocyte progenitor in human skeletal muscles.[20] To explore this aspect, mRNA expression of three markers (the UCP1 (uncoupling protein 1), the CIDEA (cell death-inducing DFFA-like effector A) and the ELOVL3 (elongase of very-long-chain fatty acids-like 3)), usually used as an index of the browning process, was analyzed. At day 0 of differentiation, *UCP1* and *CIDEA* transcripts were undetectable (Ct > 40) or were at a very low level (Ct ~ 39). When CD56$^-$ cells were cultured in adipogenic medium, both *UCP1* and *CIDEA* transcript increased by almost 200× but the level was still very low (Ct ~ 32) (Supplementary Figure S1). In contrast, adipogenic differentiation has no effect on *ELOVL3* mRNA expression. The low expression of these three genes was also observed when these cells were cultured in myogenic medium. Finally, treatment of CD56$^-$ cellular fraction at D6 of adipogenic differentiation with forskolin (1 μM, 3 h), a known stimulus of thermogenesis and browning in white adipocytes, only induced the expression of *ELOVL3* ($P<0.001$; Supplementary Figure S1). Overall, these data suggest that adipocytes derived from CD56$^-$ cellular fraction exhibit features of white rather than brown adipocytes in our culture conditions.

We next showed that cells from the CD56$^-$ fraction acquired the ability to store TG during differentiation in adipogenic medium, as suggested by BODIPY staining (Figure 1b), but also by the ability to release FA from the TG pools while stimulated with forskolin and atrial natriuretic peptide, two classical lipolytic factors in human adipocytes (Figures 3a and b). Furthermore, CD56$^-$ cells were responsive to insulin stimulation as shown by the small but significant rise in glucose uptake compared with unstimulated condition (Figure 3c). These data suggest that the CD56$^-$ cellular fraction of human skeletal muscle can differentiate into functional white adipocytes.

Investigating more deeply the immunophenotype of cells present within cultured skeletal muscle-derived SVF, we found that CD15$^+$ cell population accounts for around 20% (19 ± 7%) of the unsorted SVF, 55% (53 ± 5%) of the CD56$^-$ fraction and 5% (7 ± 2%) of the CD56$^+$ fraction (Figure 4). As expected,[9] CD15 and CD56 markers were mutually exclusive, as < 1.5% of cells expressed both markers (Figure 4). Of note, leukocytes (that is, CD45$^+$ cells) and endothelial cells (that is, CD45$^-$CD31$^+$ cells) were absent (< 0.3%) of all the fractions, probably due to culture conditions (Supplementary Figure S2). In order to better define adipogenic progenitor cell population and to further optimize the culture of human skeletal muscle-derived adipocytes, we decided to study the phenotype and functionality of CD15$^+$ cells, the major cell population of the CD56$^-$ fraction in our conditions. These cells were isolated by fluorescence-activated cell sorting and purity of the fraction was estimated at 90 ± 4%. When cultured for 7 days (D0–D6) in adipogenic medium, CD56$^-$CD15$^+$ cells acquired adipocyte-like phenotype as shown in the various panels of Figure 5. Indeed, these cells held large amount of TG-containing lipid droplets, expressed classical genes of adipocyte differentiation and were able to mobilize their TG stores on lipolytic stimulation by forskolin. As expected, *LEP* (leptine) and *PPARγ2* (peroxisome proliferator-activated receptor γ2) transcript levels were also increased as differentiation occurs ($n=5$, $P<0.05$, data not shown). Interestingly, almost all parameters analyzed here were higher in differentiated CD56$^-$CD15$^+$ fraction compared with differentiated CD56$^-$ fraction, emphasizing that CD56$^-$CD15$^+$ fraction was more concentrated in adipogenic progenitor cells. Of note, the expression profile of *UCP1*, *ELOVL3* and *CIDEA*, used as index of the browning process, was similar between CD56$^-$ and CD56$^-$CD15$^+$ fractions ($n=3$–8, $P=0.77$, data not shown). Furthermore, when compared with differentiated adipogenic progenitors from adipose tissue (human multipotent adipose-derived stem cell line (hMADS) and primary culture of abdominal dermolipectomy-derived SVF), CD56$^-$CD15$^+$-derived adipocytes had reduced mRNA levels of brown but also beige markers such as TBX1 (T-box 1 transcription factor) and CD137 (TNFRSF9 (tumor necrosis factor receptor superfamily member 9))[21] (Supplementary Figure S3). Of note, hMADS and SVFs collected from subcutaneous adipose tissue during abdominal dermolipectomy were obtained and differentiated for 10 days using standard culture protocols as previously described.[22,23]

We were able to isolate a fourth fraction, that is, the CD56$^-$CD15$^-$ fraction, in three independent experiments. Owing

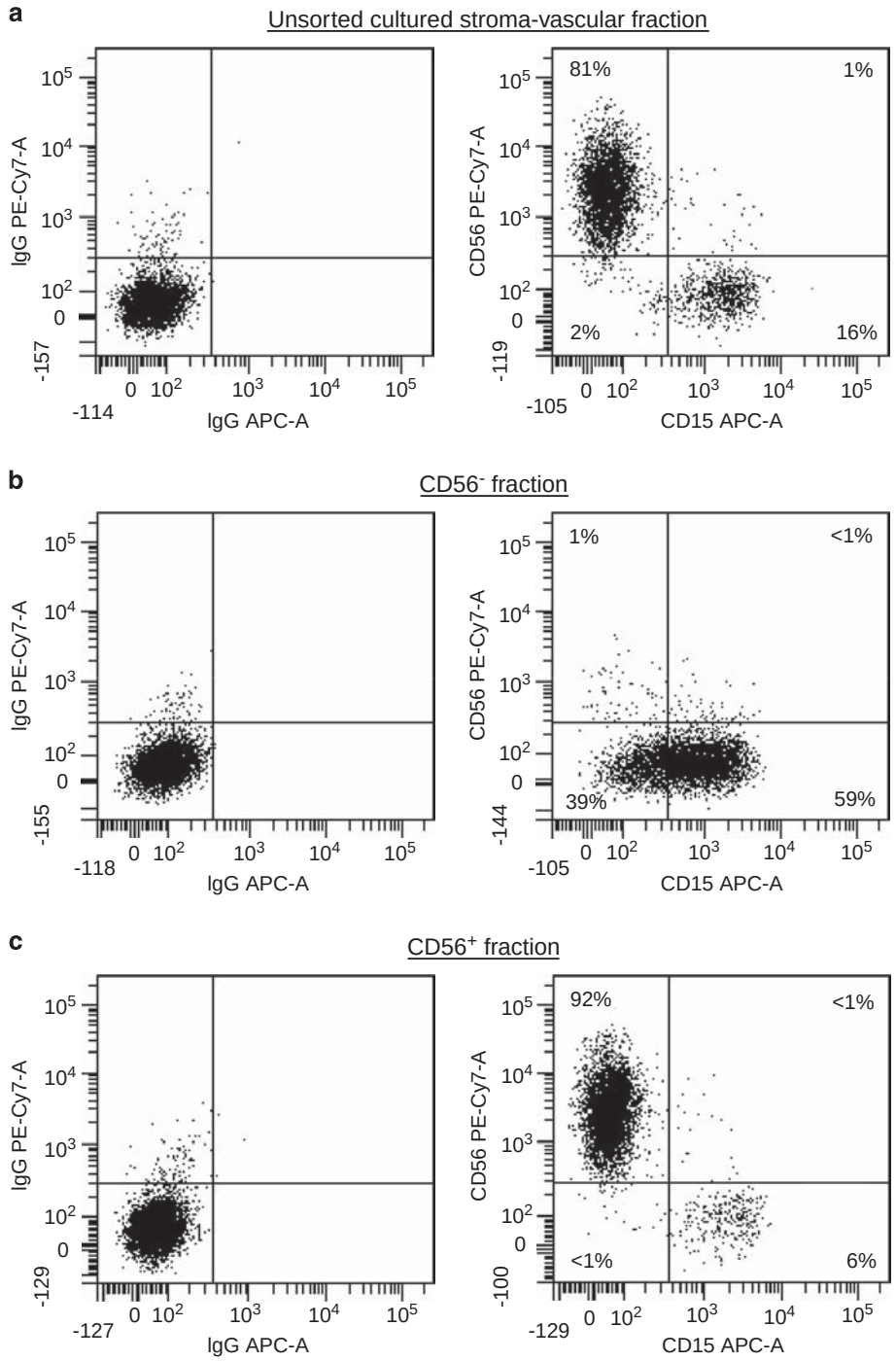

Figure 4. Cytometry analysis of skeletal muscle-derived SVF before and after cell sorting. Representative dot blots of multi-color analyses performed on cultured, unsorted SVF (**a**), $CD56^-$ cellular fraction (**b**) and $CD56^+$ cellular fraction (**c**) using antibodies directed against satellite cells ($CD56^-$PECy7) and mesenchymal stem/stromal cells (CD15-APC) or control isotypes ($n = 7–9$).

to the low rate of double negative cells, we were unable to fully investigate their phenotype but we observe using microscopy that a proportion (much lower than in the $CD56^-CD15^+$ cellular fraction) of this cellular fraction was able to adopt a round shape and store lipids when cultured in adipogenic medium. By opposition, the $CD56^-CD15^-$ fraction does not exhibit a characteristic phenotype when cultured in myogenic medium (data not shown).

To conclude, $CD15^+$ cells isolated from $CD56^-$ cellular fraction represent the major cell population within cultured human skeletal muscle-derived SVF, giving rise to white functional adipocytes.

Secretions of $CD56^-CD15^+$-derived adipocytes alter insulin signaling and action in human primary myotubes

Although IMAT content is negatively correlated with insulin resistance,[1,4] no direct data sustaining this correlation has been reported. We thus investigated the effect of skeletal muscle adipocytes, obtained from adipogenic differentiation of $CD56^-$ $CD15^+$ cells, on insulin action of human skeletal muscle fibers.

Importantly and in contrast to myotubes pre-incubated with control media (that is, not conditioned), myotubes treated with $CD56^-CD15^+$-derived adipocyte-conditioned media exhibit a

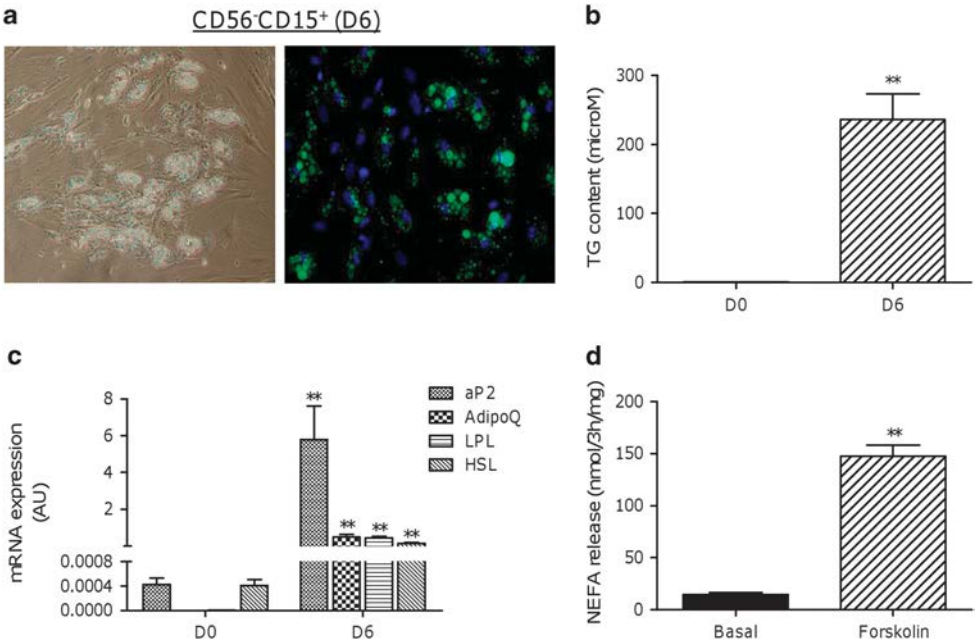

Figure 5. Phenotypical and functional analyses of $CD56^{-}CD15^{+}$ cellular fraction cultured in adipogenic conditions. (**a**) Representative optical and fluorescence microscopy picture of $CD56^{-}CD15^{+}$ cellular fraction cultured for 7 days in adipogenic medium (D6) ($n=4$; original magnification, ×100 or ×200, respectively). Nucleus was stained in blue and neutral lipids in green. (**b**) TG storage at D0 and D6 of adipogenic differentiation ($n=3$). $^{**}P<0.01$ D0 vs D6. (**c**) Quantitative PCR analysis of adipogenic-related genes at the beginning (D0) and at the end (D6) of adipogenic differentiation. ($n=4$). $^{**}P<0.01$ D0 vs D6. (**d**) Pulse-chase analysis of lipolysis at D6, measured by non-esterified fatty acid (NEFA) release from radiolabeled TG pools and performed with or without forskolin (10 μM) stimulation ($n=3$). $^{**}P<0.01$ basal vs stimulated.

strong abrogation of insulin-mediated glycogen synthesis on insulin stimulation (Figure 6a). In accordance with these data, the weak increase of glucose uptake generally observed in the presence of insulin in this *in vitro* model[24] is abolished when myotubes are pre-incubated with $CD56^{-}CD15^{+}$-conditioned media (Figure 6a). Similar results were obtained for insulin-stimulated glucose oxidation ($n=3$, data not shown). Alteration of insulin effect seems to be specific to adipocyte-derived $CD56^{-}CD15^{+}$, as conditioned media from undifferentiated $CD56^{-}CD15^{+}$ cells (D0) or $CD56^{-}CD15^{+}$ cultured for 5 days in myogenic conditions have no effect on insulin-stimulated glycogen synthesis (Supplementary Figure S4).

This was associated with a reduced insulin-stimulated phosphorylation of Akt at Serine 473 and Threonine 308 (Figure 6b). Phosphorylation of Akt at Serine 473 and Threonine 308 residues lead to Akt activation and downstream signaling (Figure 6b).

Collectively, these data indicate that adipocyte secretions derived from the $CD56^{-}CD15^{+}$ cells have a negative impact on insulin action in skeletal muscle cells.

DISCUSSION

Recent data indicate that IMAT is negatively correlated with insulin sensitivity in humans.[4,5,25] However, a causal link between the presence of intramuscular adipocytes observed in obesity and insulin resistance has not been demonstrated so far. In this study, we showed that primary culture of SVF obtained from obese human muscle biopsies contains two distinct committed progenitors that can be easily separated using the CD56 marker, a marker of satellite cells. The $CD56^{+}$ cellular fraction contains myogenic progenitors, that is, satellite cells, whereas the $CD56^{-}$ cellular fraction contains adipogenic progenitors. We next reported that the $CD56^{-}$ cellular fraction and more specifically its main constituting cell type, the $CD15^{+}$ cells, display the main morphological and metabolic features of white adipocytes. Moreover, we demonstrate for the first time that the secretions of $CD56^{-}CD15^{+}$-derived adipocytes reduce insulin-stimulated action and insulin signaling in human primary myotubes, thus providing proof-of-concept that emergence of IMAT can contribute to insulin resistance in obesity and aging.

Although it is well established that IMAT is associated with insulin resistance and an increased risk of developing type 2 diabetes,[1,2] the origin of these adipocytes and the ways they alter insulin sensitivity are not clearly established. Satellite cells, the well-known myogenic precursor, have been reported to give rise to multiple cell lineages, among them the adipogenic lineage, which may represent heterogeneity of satellite cells, multipotency or transdifferentiation of this precursor. Data from clonal analysis support the hypothesis of distinct satellite cells expressing mutually exclusive myogenic or non-myogenic (that is, adipogenic) pathways.[26,27] On the other hand, multipotency of satellite cells and transdifferentiation of the myoblast (that is, activated satellite cells) into adipocyte was reported with the C2C12 myogenic cell line[28,29] and primary culture of murine and human myoblasts.[30–32] A third possibility has recently emerged based on results obtained using plasma membrane immuno-related cell-sorting techniques. Indeed, some papers have reported that it was possible to isolate two distinct committed progenitor cells, a myogenic one, that is, the satellite cells, and an adipogenic one, also referred to fibro-adipogenic progenitors from the SVF of digested skeletal muscles.[9,10,12,33] The possibility that intramuscular adipose tissue is derived from non-myogenic lineage was supported by Cre/loxP-mediated lineage tracing in mice.[34] Different isolation methods (single fiber versus SVF associated to cell sorting) and/or culture conditions (low or high glucose, thiazolidinedione concentration or various other adipogenic factors) may explain part of the discrepancies reported above. In particular, the adipogenic potential of satellite cells obtained from single fiber isolation or SVF give rise to opposite results.[9,14,32] One explanation may come from the Cre/loxP lineage analysis recently

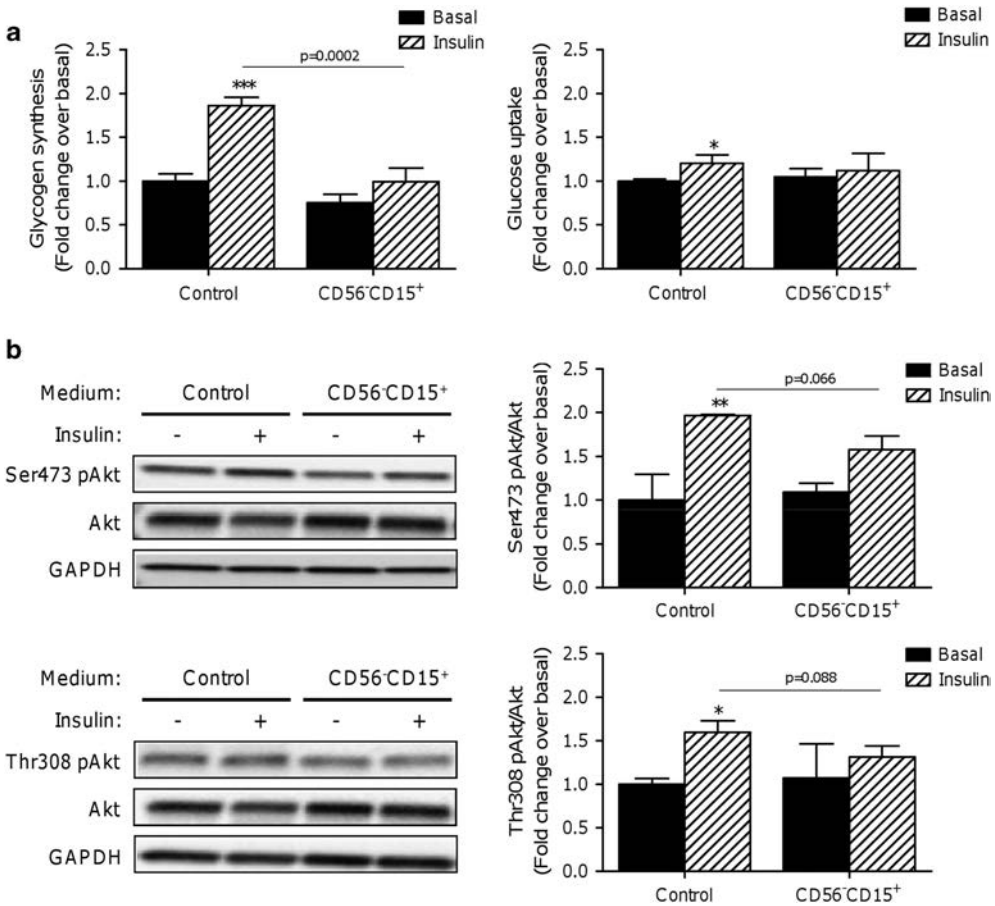

Figure 6. Insulin sensitivity of myotubes pre-incubated with differentiated $CD56^{-}CD15^{+}$-derived adipocyte-conditioned media. (**a**) Primary myotubes of lean individuals were pre-incubated with control or adipocyte-conditioned medium/DMEM, low glucose, Glutamax (v/v). Basal and insulin-stimulated glycogen synthesis and glucose uptake were then appreciated by radioactive assays. (**b**) Representative western blotting of Ser473pAkt, Thr308pAkt, Akt and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) proteins obtained from primary myotubes being treated as above. Quantification of the ratio Ser473pAkt/Akt and Thr308pAkt/Akt, two indexes of Akt activity, were then realized. (**b**) Two-way analyses of variance were performed for each studied parameter ($n = 3–5$). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ basal vs stimulated.

published by Starkey JD *et al.*[35] showing that adipocytes obtained *in vitro* were derived from contaminating cells that were unintentionally co-purified with single muscle fibers.

In accordance with the few already published data in humans[9,14] and using cell-sorting technique, we showed that two committed progenitors can be easily sorted in human skeletal muscles of obese volunteers by CD56 plasma membrane expression. Indeed, the $CD56^{+}$ cellular fraction, well known to contain the satellite cells, gave rise to myotubes independently of culture conditions, while the $CD56^{-}$ cellular fraction not only express key adipogenic program-related genes but exhibits metabolic characteristic of adipocytes when differentiated in adipogenic conditions, supporting the fact that this cellular fraction contains the adipogenic progenitors. We further observed, using genomic and metabolic phenotyping, that cells expressing the surface marker CD15 and present in $CD56^{-}$ cellular fraction are the main adipogenic progenitors. Interestingly, CD15 (also called Lewis X or SSEA-1) is a non-protein antigen corresponding to a carbohydrate residue mainly expressed by myeloid cells and widely used for the diagnosis of Hodgkin lymphoma.[36] Although few $CD15^{+}$ cells are detectable *in situ*, this marker appears in culture within hours after seeding.[9] Further experiments dedicated to fucosyltranferase expression and activity, the enzyme that synthesizes CD15, would be required to better understand the regulation and role(s) of CD15 into the adipogenic fate. These experiments would presumably explain the apparent conflicting results obtained recently by Agley CC *et al.*,[14] in which $CD15^{+}$ cells were very rarely encountered in cultured SVF of human skeletal muscle and poorly associated with adipocyte differentiation. Finally, our results reinforce the fact that $CD56^{-}CD15^{+}$-derived adipocytes acquire the phenotypic features of white adipocytes expressing low levels of both brown and beige markers. This is in agreement with previous studies.[8,12,26] It has to be noted that when $CD56^{+}$ cellular fraction was cultured in adipogenic conditions, no or very few adipocytes were observed, even when $CD15^{+}$ cell contamination was higher than a few percents, suggesting that myogenic progenitors and/or myotubes repress adipocyte differentiation. This observation is in agreement with recent data obtained in rodents.[10]

According to the present data, differentiated $CD56^{-}CD15^{+}$ cells isolated from skeletal muscles of obese individuals give rise to functional white adipocytes, secretions of which are responsible for the alteration of both insulin action and insulin signaling in human primary myotubes. In this study, insulin-stimulated glycogen synthesis was used as a main readout of insulin action, considering that human primary skeletal muscle cells exhibit a glycolytic phenotype under standard culture conditions, and that insulin displays a marginal effect on glucose uptake and oxidation in this cell model system.[24] To our knowledge, these results are the first to give a potential mechanistic explanation for the

negative correlation, frequently reported in human, between IMAT and insulin sensitivity. However, it would be interesting in the future to assess the impact of the conditioned media from differentiated adipogenic progenitors isolated from skeletal muscles of age-matched individuals with different metabolic status. Indeed, it can be hypothesized, as shown for satellite cells, that these progenitors retain characteristics of the donors.[17,37] It will also be important to identify the secreted factors involved in the effect of intramuscular adipocytes and their related molecular mechanism of action. Using large proteomic and genomic approaches, similarities but also strong differences have been reported between IMAT and other adipose tissues in pinpointing to specific features of this fat depot.[38,39] Interestingly, during the writing of the present study, Arrighi N *et al.*[40] presented data on human adipogenic progenitors from adipose tissue versus skeletal muscle, confirming that IMAT could be regarded as a specific fat compartment. However, considering the reported link between IMAT and systemic inflammation, soluble classical inflammatory mediators such as cytokines could be involved. Indeed, positive correlations between IMAT in the leg and plasma levels and/or muscle transcript levels of interleukin-6 and tumor necrosis factor-α have been reported.[6,41] In addition, using SVF of mammary fat differentiated into adipocytes, Eckardt *et al.*[42] have shown that human skeletal muscle cells undergoing co-cultures display dysfunction of insulin signaling and a reduced GLUT4 translocation to the plasma membrane. Importantly, although tumor necrosis factor-α was found to reproduce the decrease of IRS-1 and Akt phosphorylation observed with co-cultures, it was undetectable in the mammary-derived adipocyte-conditioned media,[43] suggesting that other adipo/cytokines (leptin, monocyte chemotactic protein-1, interleukin-8 and so on) are involved.[44,45] Besides adipo/cytokines, lipolysis-derived free FA and other lipid derivatives have been shown to induce skeletal muscle insulin resistance.[44,46] Interestingly, Kovalik JP *et al.*[47] reported that co-culture with subcutaneous differentiated adipocyte progenitors forces myotubes to use FA rather than glucose and remarkably increases intramyocellular TG content with prolonged exposure. This was associated with significant reduction of insulin signaling. Those data are in accordance with the negative correlation reported *in vivo* between intramyocellular TG content and insulin sensitivity.[48–50] However, diacyglycerols and ceramides, two lipid derivatives that are particularly linked to alteration of insulin signaling,[51] were not increased. In contrast, the acylcarnitine profile was changed, supporting connection between skeletal muscle insulin resistance and lipid-induced mitochondrial stress.[52] These observations suggest that trafficking of FA from IMAT to intramyocellular TG could contribute to insulin resistance and metabolic dysfunction of skeletal muscle fiber in the obese state and would require further investigations.

In summary, we report here that cultured SVF obtained from skeletal muscle of obese donors contains committed adipogenic progenitors, with immunophenotype $CD56^{-}CD15^{+}$, able to differentiate into functional white adipocytes in culture. Secretions of these adipocytes reduced insulin action and signaling in primary myotubes from lean donors, suggesting that IMAT may contribute to the development of skeletal muscle insulin resistance in a paracrine manner. To the best of our knowledge, these are the first *in-vitro* data supporting a causal and mechanistic link between IMAT and insulin resistance. These findings open interesting perspectives for future studies, to better understand the cross-talk between intramuscular adipocytes and myofibers within human skeletal muscle in the context of obesity and aging.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on International Journal of Obesity website (http://www.nature.com/ijo)

Supplemental Table 1. Details of culture conditions.

Name	Composition	Duration
Proliferation medium 1	DMEM (Dulbecco's modified Eagles's medium) low glucose-Glutamax™, 10% fetal bovine serum (FBS), human epithelial growth factor (hEGF, 10 ng/ml), dexamethasone (0.39 µg/ml), BSA (0.05%), fetuin (0.5 mg/ml), gentamycin (50 µg/ml) and fungizone (50 ng/ml),	Until 80% to 100% confluence
Proliferation medium 2	DMEM low glucose-Glutamax™, 10% FBS, fibroblast growth factor 2 (FGF2, 2.5 ng/ml), dexamethasone (0.39 µg/ml), gentamycin (50 µg/ml) and fungizone (50 ng/ml)	Until 80% to 100% confluence
Myogenic medium	α-MEM (Minimum Essential Medium Eagle) low glucose-Glutamax™ , 2% penicillin-streptomycin, 2% FBS and fetuin (0.5 mg/ml)	For 5 days
Adipogenic medium	DMEM low glucose-Glutamax™/HAMF12 (v/v), 2% FBS, gentamycin (50 µg/ml), Biotin (8 µg/ml), D-panthotenate (4 µg/ml), transferrin (10 µg/ml), insulin (66 nM), triiodothyronin (T3, 1 nM), cortisol (0.1 µM) and rosiglitazone (1 µM)	For 7 days

Supplemental Table 2. List of human primer and probe sequences used for real-time qPCR

Gene symbol	**Taqman Probe ID**
PAX7 (Paired box 7)	Hs00242962_m1
MYH7 (myosin heavy chain 7)	Hs01110632_m1
MYOG (myogenin)	Hs01072232_m1
MYF5 (myogenic factor 5)	Hs00271574_m1
HSL (hormone-sensitive lipase)	Hs00943404_m1
LPL (lipoprotein lipase)	Hs00173425_m1
aP2 (fatty acid binding protein 4)	Hs01086177_m1
AdipoQ (adiponectin)	Hs00605917_m1
LEP (leptin)	Hs00174877_m1
TXB1 (T-box 1)	Hs00962556_m1
CD137 (TNFRSF9, tumor necrosis factor receptor superfamily, member 9)	Hs00155512_m1
UCP1 (uncoupling protein 1)	Hs00222453_m1
ELOVL3 (elongation of very long chain fatty acid-like 3)	Hs00537016_m1
*CIDEA (*cell death-inducing DFFA-like effector A)	Hs00154455_m1
18S	Hs99999901_s1

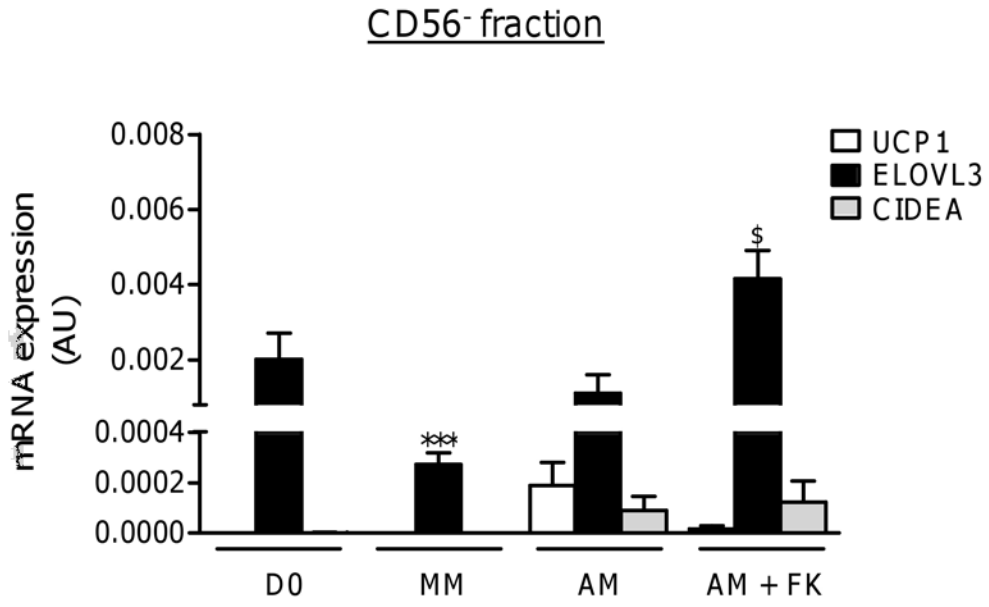
Gene symbol	**Forward**	**Reverse**
PPARγ2 (peroxisome proliferator-activated receptor gamma 2)	CAAACCCCTATTCCATGCTGTT	ATCAGTGAAGGAATCGCTTTCTG

Supplemental Figure 1. Gene expression of brown markers of $CD56^-$ cellular fraction. *UCP1*, *ELOVL3* and *CIDEA* transcript levels, known to be increased during browning, were evaluated by qPCR in (i) undifferentiated $CD56^-$ cells (D0); (ii) $CD56^-$ cells cultured in myogenic medium for five days (MM); (iii) $CD56^-$ cells cultured in adipogenic medium for seven days (AM) stimulated or not for 3 h with forskolin (FK, 1 µM). Two-way ANOVA analyses (D0 *vs* MM; D0 *vs* AM, AM *vs* AM + FK) were performed. (n=4 to 8). $^{***}p<0.001$ D0 *vs* MM; $ p<0.001 AM *vs* AM + FK.

Supplemental Figure 2.. Cytometry analysis of skeletal muscle-derived stroma-vascular fraction before and after cell sorting. Representative dot blots of multi-color analyses performed on cultured unsorted stroma-vascular fraction (A), $CD56^-$ cellular fraction (B) and $CD56^+$ cellular fraction (C) using antibodies directed against leukocytes (CD45-PerCP) and endothelial cells (CD31-FITC) or control isotypes (n=7 to 9).

Supplemental Figure 3. Comparison of brown and beige expression markers of $CD56^-$ $CD15^+$ cellular fraction, hMADS and adipose-tissue-derived SVF cultured in adipogenic conditions. *UCP1*, *ELOVL3* and *CIDEA* transcript levels (brown adipocyte markers) and *CD137* and *TBX1* (beige adipocyte markers) were evaluated by qPCR. cDNA from differentiated hMADS (Human multipotent adipose-derived stem cells) and SVF from abdominal dermolipectomy (ADL) of obese subjects (4 females, age: 44±3.9 years, BMI: 30±0.9 kg/m²) was taken from our in house biobank and compared to cDNA of differentiated $CD56^-CD15^+$ cells. One way ANOVA analyses were performed for each gene and revealed statistically differences between groups for UCP1 and CD137 expression and a tendency for CIDEA and TBX1 (n=3 to 8). $^{*}p<0.05$.

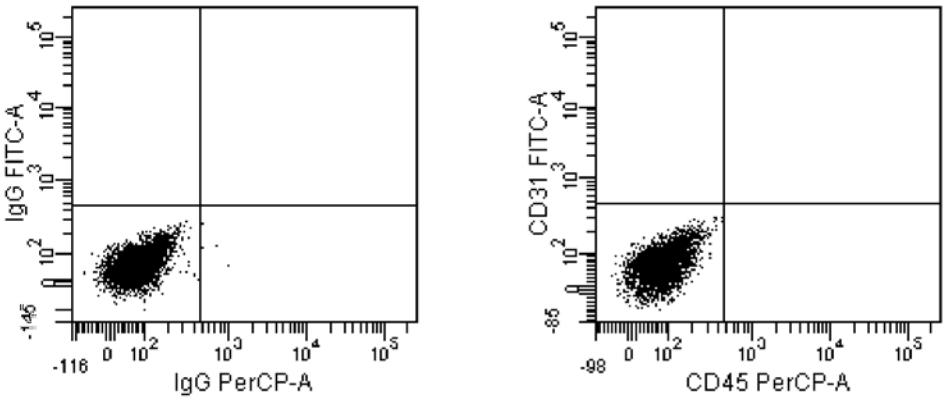
Supplemental Figure 4. Insulin-stimulated glycogen synthesis of myotubes pre-incubated with conditioned media from undifferentiated or differentiated $CD56^{-}CD15^{+}$ cells. $CD56^{-}CD15^{+}$ cells were cultured 24 h in DMEM low glucose-Glutamax™ with free fatty acid BSA 0.4 % and PS 1% before induction of differentiation (D0), after 5 days of differentiation in myogenic medium (MM) or after 7 days of differentiation in adipogenic medium (AM). Primary myotubes (D4) of lean individuals were then pre-incubated with $CD56^{-}CD15^{+}$ cells-conditioned media/DMEM low glucose-Glutamax™ (v/v) for an overnight period (18 h). Insulin-stimulated glycogen synthesis was determined as described in Material and Methods section. One way ANOVA analysis was performed and revealed differences between groups (n=3 to 6).* $p<0.05$ and $^{***}p<0.001$.



Supplemental Figure 1

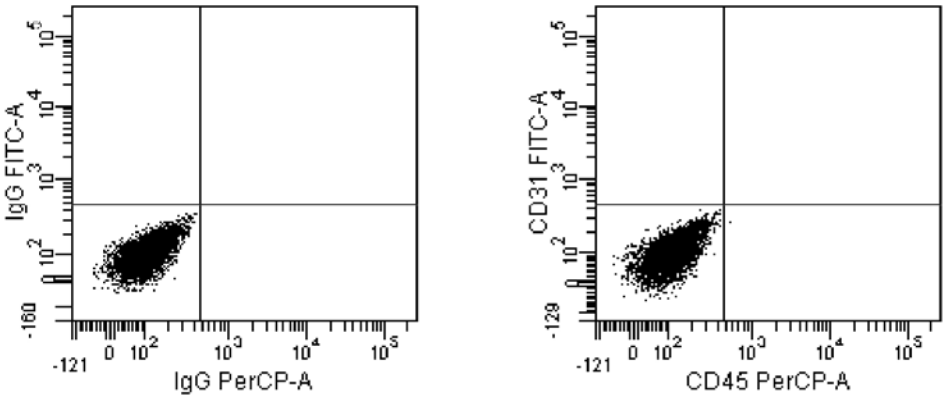
A

Unsorted cultured stroma-vascular fraction



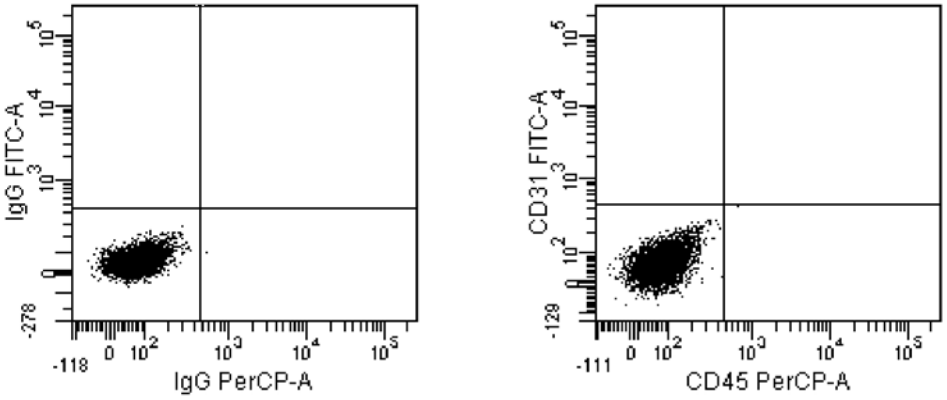
B

CD56⁻ fraction

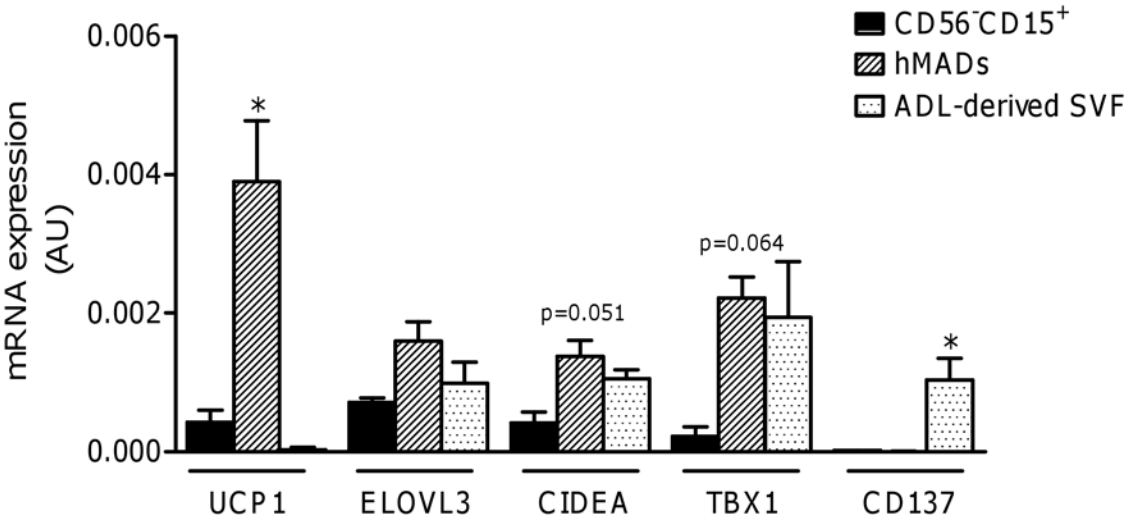


C

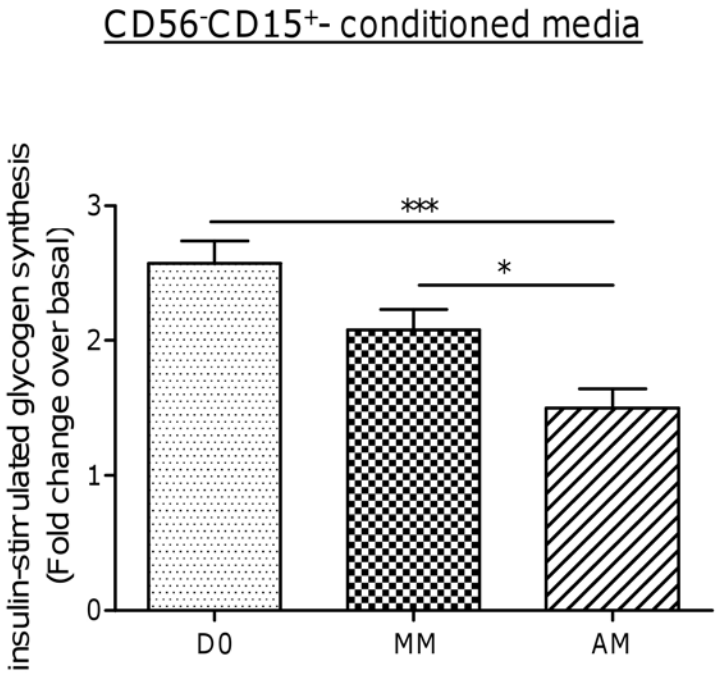
CD56⁺ fraction



Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4

8.1.2. Discussion

Bien que la présence d'un dépôt adipeux au sein du muscle squelettique soit connue de longue date, l'origine cellulaire (FAP, fibrocytes, ASC, cellules satellites…) de l'IMAT fait encore débat de nos jours. Par ailleurs, de nombreuses études ont associé l'accumulation d'IMAT et le développement d'une insulino-résistance musculaire et systémique (Boettcher *et al.*, 2009; Goodpaster *et al.*, 2000; Ryan and Nicklas, 1999; Yim *et al.*, 2007), bien qu'aucun lien causal n'ait été mis en évidence. ***Ce premier travail a donc consisté à confirmer, d'une part, qu'il existe au sein du muscle squelettique de sujets obèses une population de cellules progénitrices, différente des cellules satellites, capable de donner naissance à des adipocytes matures en culture, et de définir les caractéristiques de ces adipocytes (i.e. propriétés métaboliques, phénotype d'adipocytes blanc, beiges ou bruns) et à établir si, d'autre part, les sécrétions de ces adipocytes peuvent altérer la sensibilité à l'insuline des fibres musculaires.***

Il a récemment été décrit que le muscle squelettique humain contient au moins deux populations de cellules progénitrices. La première population est caractérisée par l'expression du marqueur de surface CD56 et présente un fort potentiel de différenciation myogénique *in vitro*, il s'agit des cellules satellites. La deuxième population est dépourvue du marqueur CD56 mais exprime le marqueur CD15 (population $CD56^{-}CD15^{+}$), et est capable de donner naissance à des adipocytes *in vitro*, il s'agit des progéniteurs fibro/adipogéniques (FAP) (Joe *et al.*, 2010; Lecourt *et al.*, 2010; Pisani *et al.*, 2010b; Uezumi *et al.*, 2010). Ces études menées chez l'homme ont été réalisées à partir de biopsies musculaires d'individus sains/normo-pondérés ou présentant des myopathies, et se sont davantage intéressées à l'étude du lignage de ces progéniteurs adipocytaires (mar l'étude des marqueurs de surface) qu'aux propriétés métaboliques et fonctionnelles des adipocytes dérivés des FAP. Par ailleurs, l'utilisation du marqueur de surface CD15 comme marqueur spécifique des FAP est encore débattue. En effet, et bien qu'il ait été mis en évidence que ce marqueur est peu présent dans le muscle squelettique *in vivo*, et est induit après quelques heures de culture (Lecourt *et al.*, 2010), une étude plus récente a démontré que les cellules $CD15^{+}$ sont très rares parmi les cellules présentes dans des cultures de SVF musculaire humaine, et ne se différencient pas en adipocytes lorsqu'elles

sont cultivées en conditions adipogéniques (Agley *et al.*, 2013). Enfin, il a également été décrit que les cellules satellites sont capables de se différencier non seulement en fibres musculaires squelettiques, mais aussi en d'autres types cellulaires, notamment en ostéoblastes, cellules musculaires lisses et adipocytes. Il a été proposé que cela puisse être dû soit à une hétérogénéité des cellules satellites, soit à leur multipotence ou bien encore à une capacité de trans-différenciation de ces cellules. Ces hypothèses sont toujours controversées au sein de la communauté scientifique. Il apparaît donc d'après ce résumé de la littérature que de nombreux points restent à éclaircir concernant l'origine cellulaire des adipocytes musculaires ainsi que leurs propriétés métaboliques.

Nous montrons dans cet article que les cellules de la fraction $CD56^+$, contenant les cellules satellites, sont capables de se différencier en myotubes, et que les cellules de la fraction $CD56^-$, lorsqu'elles sont cultivées dans un milieu adipogénique, expriment les gènes classiquement induits lors de l'adipogenèse et présentent les caractéristiques métaboliques d'adipocytes matures. Ces résultats suggèrent donc que la fraction $CD56^-$ contient les cellules progénitrices adipogéniques. Par ailleurs, il est important de noter que les cellules de la fraction $CD56^+$ ne sont capables de se différencier qu'en myotubes, qu'elles soient cultivées dans un milieu de différenciation myogénique ou adipogénique, et que les cellules de la fraction $CD56^-$ se différencient en adipocytes en milieu adipogénique, mais ne sont pas capables de se différencier en myotubes lorsqu'elles sont cultivées en milieu myogénique, confirmant ainsi l'existence de deux progéniteurs distincts engagés soit dans le lignage myogénique soit dans le lignage adipogénique, comme précédemment rapporté (Joe *et al.*, 2010; Lecourt *et al.*, 2010; Pisani *et al.*, 2010b; Uezumi *et al.*, 2010). De plus, nous avons observé, par des approches d'expression génique et de phénotypage métabolique, que les cellules exprimant le marqueur de surface CD15 sont les progéniteurs adipogéniques majoritaires au sein de la fraction $CD56^-$ (Figure 16), en accord avec certaines études (Joe *et al.*, 2010; Lecourt *et al.*, 2010; Pisani *et al.*, 2010b; Uezumi *et al.*, 2010), mais en désaccord avec les résultats obtenus par C.C. Agley *et al.* (Agley *et al.*, 2013). Il est possible d'imaginer que l'expression du marqueur CD15 à la surface des FAP puisse être modulée par des différences dans les conditions de culture de ces cellules (*i.e.* densité

d'ensemencement, type de support des plaques de culture) ou dans la composition des milieux de culture, expliquant ainsi ces résultats à priori contradictoires.

Pour compléter la caractérisation des adipocytes dérivés des FAP, nous avons voulu déterminer si les cellules $CD56^{-}CD15^{+}$ présentaient le phénotype d'adipocytes blancs ou bruns. En effet, il a été décrit que des progéniteurs d'adipocytes bruns sont présents au sein du muscle squelettique (Almind *et al.*, 2007; Crisan *et al.*, 2008). Nous avons constaté, en accord avec d'autres études (Arrighi *et al.*, 2015; Pisani *et al.*, 2010b), que ces cellules acquièrent en culture le phénotype d'adipocytes blancs, et les marqueurs classiques d'adipocytes bruns et beiges y sont exprimés à très faible niveau.

Enfin, et de façon intéressante, nous avons constaté que lorsque les cellules $CD56^{+}$ sont cultivées en condition adipogénique, y compris lorsque des cellules contaminantes $CD15^{+}$ sont présentes, peu ou pas d'adipocytes sont présents dans la culture. Ces résultats suggèrent que, comme cela a été observé chez le rongeur (Uezumi *et al.*, 2010), les cellules progénitrices musculaires et/ou les myotubes seraient capables d'inhiber la différenciation adipocytaire.

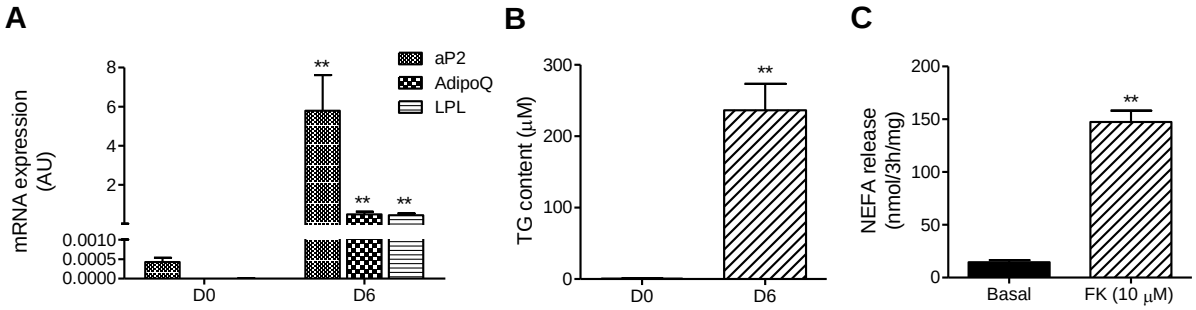


Figure 16. Caractérisation des cellules $CD56^{-}$ $CD15^{+}$ cultivées en conditions adipogéniques

(A) Expression de gènes de l'adipogenèse au début (D0) et à la fin (D6) de la différenciation adipogénique (n=4). **p<0.01 D0 vs D6. (B) Stockage de TG en début (D0) et fin (D6) de différenciation adipogénique (n=3). **p<0.01 D0 vs D6. (C) Mesure de la lipolyse en fin de différenciation (D6) évaluée par la quantification des AG libres dans le milieu de culture (NEFA : non-esterified fatty acids) en absence (Basal) ou présence (FK) de 10µM de forskoline (n=3). **p<0.01 basal vs FK.

Nous avons dans un second temps montré que les adipocytes issus des cellules $CD56^{-}CD15^{+}$ isolées de biopsies musculaires de sujets obèses sont capables de sécréter des facteurs responsables d'une altération de la sensibilité à l'insuline des myotubes humains en culture primaire, reflétée par une diminution de la synthèse de glycogène et de la phosphorylation d'Akt stimulées par l'insuline (Figure 17).

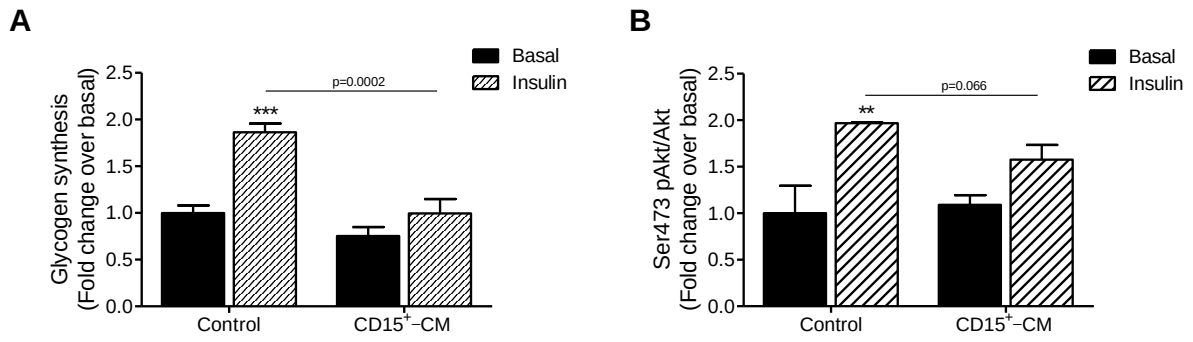


Figure 17. Altération de la sensibilité à l'insuline de myotubes pré-incubés avec du milieu conditionné de cellules $CD56^{-}$ $CD15^{+}$ différenciés en adipocytes

(A) Mesure de la synthèse de glycogène en conditions basales et stimulées par l'insuline dans des myotubes pré-incubés avec du milieu contrôle ou du milieu conditionné de cellules CD56-CD15+ différenciées en adipocytes (B) Quantification de la phosphorylation activatrice d'Akt sur le résidu ser473 en conditions basales et stimulées par l'insuline dans des myotubes pré-incubés avec du milieu contrôle ou du milieu conditionné de cellules CD56-CD15+ différenciées en adipocytes. n=3-5. **p<0.01, ***p<0.001 basal vs insulin.

Il sera important dans le futur de confirmer cette relation *in vivo* et d'identifier le/les facteur(s) responsable(s) de cette altération de la sensibilité à l'insuline musculaire et de définir leur mécanisme d'action. Il est possible d'imaginer, étant donné la corrélation positive décrite entre l'accumulation d'IMAT et le développement d'une inflammation systémique, que des médiateurs inflammatoires solubles, tels que l'IL-6 ou le TNF-α, puissent être impliqués dans l'altération de la sensibilité à l'insuline des fibres musculaires (Addison *et al.*, 2014; Beasley *et al.*, 2009). Il est par ailleurs connu que les AG issus de la lipolyse et d'autres dérivés lipidiques, tels que les DAG et les céramides, sont également capables d'induire une insulino-résistance musculaire (Lee *et al.*, 2009; Samuel and Shulman, 2012). Il a ainsi été mis en évidence que, lorsqu'ils sont co-cultivés avec des adipocytes primaires obtenus *in vitro*, issus de tissu adipeux et non de muscle, les myotubes utilisent

préférentiellement des AG comme substrat énergétique, stockent davantage d'IMTG et présentent une diminution de la signalisation insulinique (Kovalik *et al.*, 2011). Cependant, aucune augmentation des niveaux intracellulaires d'intermédiaires lipotoxiques tels que les diacylglycérols ou les céramides n'a été observée dans ces conditions. En revanche, des modifications du profil d'acylcarnitines ont été observées, suggérant une implication du stress mitochondrial induit par une surcharge lipidique dans le développement de l'insulino-résistance des cellules musculaires (Koves *et al.*, 2008). Ces résultats suggèrent donc que le stockage d'AG issus de l'IMAT au sein des IMTG des fibres musculaires pourrait contribuer à l'altération de la sensibilité à l'insuline au sein du muscle squelettique.

En résumé, ce travail démontre que le muscle squelettique de sujets obèses contient des progéniteurs adipocytaires, distincts des progéniteurs myocytaires que sont les cellules satellites, capables de se différencier en adipocytes blancs matures *in vitro*. Ces résultats mettent pour la première fois en évidence un rôle causal des adipocytes intramusculaires dans l'altération de la sensibilité à l'insuline des fibres musculaires (figure 18), et contribuent ainsi à améliorer notre compréhension de l'association observée entre IMAT et insulino-résistance chez l'homme. Les futurs travaux de recherche devront s'attacher à mieux comprendre les mécanismes moléculaires impliqués dans ce dialogue entre IMAT et fibres musculaires, notamment dans un contexte d'obésité et de diabète de type 2.

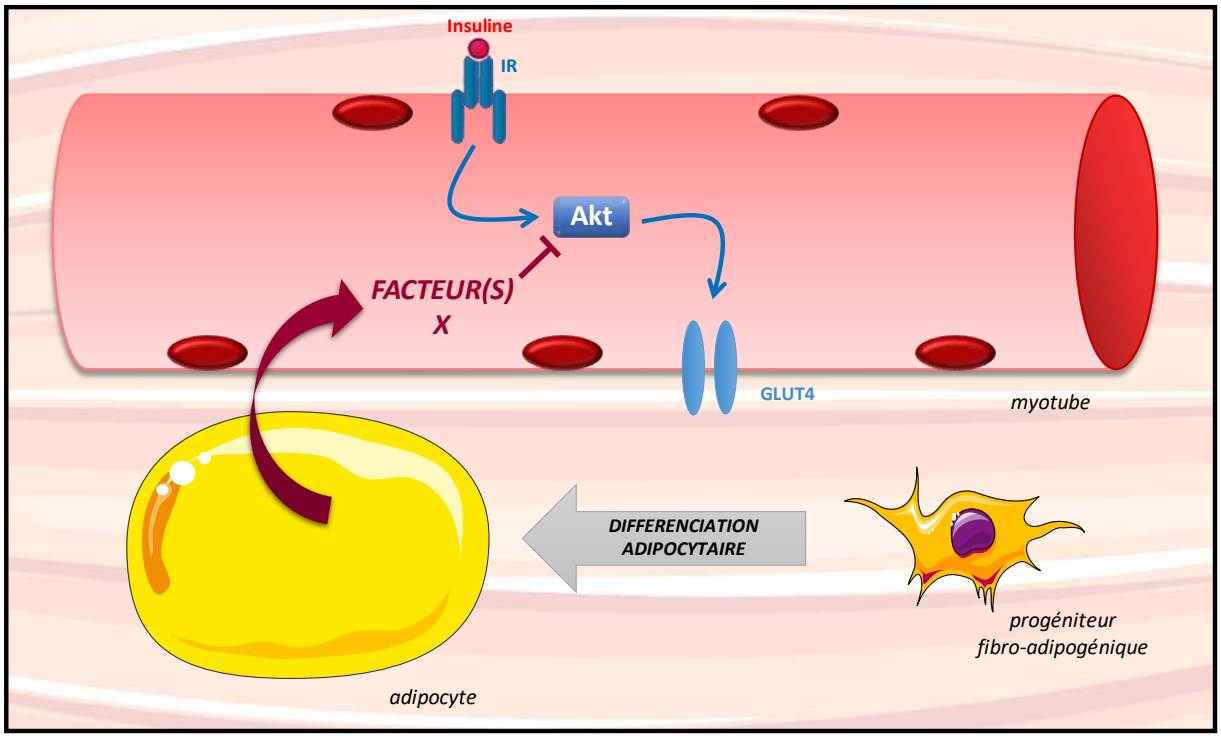


Figure 18. Modèle représentant l'impact des progéniteurs fibro/adipogéniques différenciés en adipocytes sur les fibres musculaires

Les progéniteurs fibro/adipogénique différenciés en adipocytes blancs sont capables de sécréter des facteurs, non identifiés à l'heure actuelle, responsables d'une altération de la signalisation insulinique dans les fibres musculaires, notamment via une diminution des niveaux de phosphorylations activatrices d'Akt. IR : récepteur à l'insuline ; GLUT4 : *glucose transporter 4*

8.2. PUBLICATION 2 : G0/G1 SWITCH GENE 2 CONTROLE L'ACTIVITE DE L'ADIPOSE TRIGLYCERIDE LIPASE ET LE METABOLISME LIPIDIQUE DANS LE MUSCLE SQUELETTIQUE

G0S2 est une protéine de 11KDa identifiée en 1991 comme pouvant jouer un rôle dans le cycle cellulaire, notamment dans le passage de la phase G_0 à la phase G_1 dans des lymphocytes en culture (Russell and Forsdyke, 1991). Depuis, de nombreuses études ont mis en évidence un rôle soit pro-prolifératif soit anti-prolifératif de G0S2 en fonction des types cellulaires (Heckmann *et al.*, 2013). Ce n'est qu'en 2005 qu'un rôle métabolique a été suggéré pour G0S2, lorsqu'il a été décrit comme gène cible des *Peroxysome Proliferator-Activated Receptors* (PPAR), acteurs clé de la régulation du métabolisme énergétique, dans des hépatocytes primaires de souris (Zandbergen *et al.*, 2005). Récemment, G0S2 a été identifié comme capable d'inhiber l'activité de l'ATGL dans le tissu adipeux (Schweiger *et al.*, 2012; Yang *et al.*, 2010) et le foie (Wang *et al.*, 2013; Zhang *et al.*, 2014).

Dans ce travail, nous nous sommes intéressés au rôle de G0S2 dans le contrôle du métabolisme lipidique musculaire *in vitro* chez l'homme et *in vivo* chez la souris. Par ailleurs, étant donné que des dérégulations de la lipolyse musculaire sont impliquées dans le développement de l'insulino-résistance, nous avons évalué l'impact d'une invalidation de G0S2 dans le muscle squelettique *in vivo* chez la souris sur la sensibilité à l'insuline musculaire.

8.2.1. Publication 2

G0 / G1 Switch Gene 2 controls adipose triglyceride lipase activity and lipid metabolism in skeletal muscle

Claire Laurens, Pierre-Marie Badin, Katie Louche, Aline Mairal, Geneviève Tavernier, André Marette, Angelo Tremblay, S. John Weisnagel, Denis R. Joanisse, Dominique Langin, Virginie Bourlier and Cedric Moro

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Original article

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G0/G1 Switch Gene 2 controls adipose triglyceride lipase activity and lipid metabolism in skeletal muscle

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ABSTRACT

Objective: Recent data suggest that adipose triglyceride lipase (ATGL) plays a key role in providing energy substrate from triglyceride pools and that alterations of its expression/activity relate to metabolic disturbances in skeletal muscle. Yet little is known about its regulation. We here investigated the role of the protein G0/G1 Switch Gene 2 (G0S2), recently described as an inhibitor of ATGL in white adipose tissue, in the regulation of lipolysis and oxidative metabolism in skeletal muscle.

Methods: We first examined G0S2 protein expression in relation to metabolic status and muscle characteristics in humans. We next overexpressed and knocked down G0S2 in human primary myotubes to assess its impact on ATGL activity, lipid turnover and oxidative metabolism, and further knocked down G0S2 *in vivo* in mouse skeletal muscle.

Results: G0S2 protein is increased in skeletal muscle of endurance-trained individuals and correlates with markers of oxidative capacity and lipid content. Recombinant G0S2 protein inhibits ATGL activity by about 40% in lysates of mouse and human skeletal muscle. G0S2 overexpression augments (+49%, $p < 0.05$) while G0S2 knockdown strongly reduces (−68%, $p < 0.001$) triglyceride content in human primary myotubes and mouse skeletal muscle. We further show that G0S2 controls lipolysis and fatty acid oxidation in a strictly ATGL-dependent manner. These metabolic adaptations mediated by G0S2 are paralleled by concomitant changes in glucose metabolism through the modulation of *Pyruvate Dehydrogenase Kinase 4* (PDK4) expression (5.4 fold, $p < 0.001$). Importantly, downregulation of G0S2 *in vivo* in mouse skeletal muscle recapitulates changes in lipid metabolism observed *in vitro*.

Conclusion: Collectively, these data indicate that G0S2 plays a key role in the regulation of skeletal muscle ATGL activity, lipid content and oxidative metabolism.

Keywords Lipid metabolism; Skeletal muscle; Lipolysis; Adipose triglyceride lipase; Oxidative metabolism

1. INTRODUCTION

Obesity is one of the most prevalent diseases worldwide and constitutes a major risk factor for the development of type 2 diabetes [1,2]. Over the past few decades, efforts have been made to understand how alterations in lipid metabolism can lead to the development of insulin resistance [3]. A common feature of obesity and type 2 diabetes is ectopic lipid storage. Indeed, in conditions of excess body fat and/or dietary lipid intake, fatty acids are stored as triacylglycerols within lipid droplets in adipose and non-adipose tissues such as liver, heart and skeletal muscle [4].

Interestingly, it has been repeatedly observed that intramyocellular triacylglycerol (IMTG) accumulation predicts the development of insulin resistance [5–7]. However, this relationship has proven much more complex than initially thought. Indeed, the skeletal muscle of endurance-trained athletes is highly insulin-sensitive despite having an elevated IMTG content [8]. This may be explained by a more efficient coupling between fatty acid storage and utilization in muscles from trained subjects. On the contrary, defective lipid handling in skeletal muscle has been reported in obese and type 2 diabetic subjects, associated with the accumulation of lipotoxic lipid species, such as diacylglycerols and ceramides, that impair insulin signaling and action [9,10]. We and others recently observed that disturbances of lipolysis and lipid droplet dynamics in skeletal muscle relate to lipotoxicity and insulin resistance [11–13]. The first and rate-limiting step of skeletal muscle lipolysis is catalyzed by adipose triglyceride lipase (ATGL), and

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[8] Virginie Bourlier, Cedric Moro contributed equally to this work.

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elevated expression and activity of ATGL causes insulin resistance *in vitro* in primary skeletal muscle cells [11]. However, little is known about its regulation in skeletal muscle. We showed in a previous study that Comparative Gene Identification-58 (CGI-58) co-activates ATGL in skeletal muscle [14]. *G0/G1 Switch Gene 2* encodes for an 11 KDa protein (i.e. G0S2) discovered in 1991 to be induced during the transition from the G0 to G1 phase of the cell cycle in lymphocytes [15]. In 2005, a metabolic role of G0S2 was suggested when it was identified as a Peroxisome-Proliferator-Activated Receptor (PPAR) target gene [16]. Recently, G0S2 was shown to inhibit ATGL activity in metabolic organs such as adipose tissue [17,18] and liver [19,20].

In the present study, we investigated the role of G0S2 in the control of ATGL activity and lipid metabolism in mouse and human skeletal muscle. The functional role of G0S2 in the regulation of lipolysis and energy substrate oxidation was studied through gain and loss of function studies *in vitro* in human primary muscle cells and *in vivo* in mouse skeletal muscle.

2. MATERIALS AND METHODS

2.1. Muscle sampling

Data and samples from 50 men aged between 34 and 53 years were available from a prior study [21]. Of these, 11 were normal weight sedentary controls (mean age 44.4 $\pm$ 1.1 yrs; mean BMI 23.9 $\pm$ 0.5 kg m^{-2}), 11 were normal weight endurance-trained individuals (mean age 47.9 $\pm$ 1.8 yrs; mean BMI 23.4 $\pm$ 0.4 kg m^{-2}), and others were sedentary obese (mean age 43.2 $\pm$ 1.1 yrs; mean BMI 34.0 $\pm$ 0.8 kg m^{-2}) (n = 28). The overall study design and subject testing have been partly described in [21]. The study was performed according to the latest version of the Declaration of Helsinki and the Current International Conference on Harmonization (ICH) guidelines. The research protocol was approved by the Université Laval ethics committee, and all subjects provided written informed consent. Samples of *vastus lateralis* ($\sim$100 mg) were obtained, blotted free of blood, cleaned to remove fat and connective tissue, and snap-frozen in liquid nitrogen for lipid and Western blot analyses. All samples were stored at -80 °C under argon or nitrogen gas until use.

2.2. Skeletal muscle primary cell culture

Chemicals and culture media were from Sigma–Aldrich and Life Technologies.

Satellite cells from *rectus abdominis* of healthy male subjects (age 34.3 $\pm$ 2.5 years, BMI 26.0 $\pm$ 1.4 kg/m^2, fasting glucose 5.0 $\pm$ 0.2 mM) were kindly provided by Prof. Arild C. Rustan (Oslo University, Norway). Satellite cells were isolated by trypsin digestion, preplated on an uncoated petri dish for 1 h to remove fibroblasts, and subsequently transferred to T-25 collagen-coated flasks in Dulbecco's Modified Eagle's Medium (DMEM) low glucose (1 g/L) supplemented with 10% FBS and various factors (human epidermal growth factor, BSA, dexamethasone, gentamycin, fungizone, fetuin) as previously described [22]. Cells from several donors were pooled and grown at 37 °C in a humidified atmosphere of 5% CO_2. Differentiation of myoblasts (i.e. activated satellite cells) into myotubes was initiated at $\sim$80–90% confluence by switching to α-Minimum Essential Medium with 2% penicillin-streptomycin, 2% FBS, and fetuin (0.5 mg/ml). The medium was changed every other day, and cells were grown up to 5 days.

2.3. Overexpression and knockdown studies

For overexpression experiments, adenoviruses expressing in tandem GFP and human G0S2 (hG0S2) were used (Vector Biolabs, Philadelphia, PA). Control was performed using adenoviruses containing GFP gene only. Myotubes were infected with both adenoviruses at day 4 of differentiation and remained exposed to the virus for 24 h in serum-free DMEM containing 100 μM of oleate complexed to BSA (ratio 2/1). For knockdown studies, myoblasts were exposed for 24 h at the beginning of the differentiation to lentiviral particles encoding for hG0S2 shRNA, hATGL shRNA or a scramble shRNA (non-target control) (Sigma–Aldrich, France). Oleate was preferred to palmitate for lipid loading of the cells to favor triacylglycerol (TAG) synthesis and to avoid the intrinsic lipotoxic effect of palmitate [23].

2.4. Animal studies

All experimental procedures were approved by a local ethical committee and performed according to INSERM animal core facility guidelines and to the 2010/63/UE European Directive for the care and use of laboratory animals. Eight-week-old C57BL/6J male mice were housed in a pathogen-free barrier facility (12 h light/dark cycle) and fed normal chow diet (10% calories from fat) (D12450B, Research Diets, New Jersey). After 6 weeks of diet, mice were injected with 1×10^{10} GC (i.e. genome copy) of AAV1 vector (Vector Biolabs, Philadelphia, PA) in *tibialis anterior* and *gastrocnemius* muscles. Each mouse had one leg injected with AAV1-shG0S2 and the contralateral leg injected with AAV1-shNT (nontarget) as a control. Six weeks following the injections, mice were killed by cervical dislocation and muscles (i.e. *tibialis anterior*, *gastrocnemius*, *extensor digitorum longus* and *soleus*) were dissected and either used *ex-vivo* for palmitate oxidation assay or stored at -80 °C for protein and RNA analyses.

2.5. Real-time RT-qPCR

Total RNA from cultured myotubes or *tibialis anterior* muscle was isolated using Qiagen RNeasy mini kit according to manufacturer's instructions (Qiagen GmbH, Hilden, Germany). The quantity of RNA was determined on a Nanodrop ND-1000 (Thermo Scientific, Rockford, IL, USA). Reverse transcriptase PCR was performed on a Techne PCR System TC-412 using the Multiscribe Reverse Transcriptase method (Applied Biosystems, Foster City, CA). Real-time quantitative PCR (qPCR) was performed to determine cDNA content. All primers were bought from Applied Biosystems. Primers used were: 18S (Taqman assay ID: Hs99999901_s1), G0S2 (Hs00274783_s1 and Mm00484537_g1), PDK4 (Hs01037712_m1), and PGC1α (Hs00173304_m1). The amplification reaction was performed in duplicate on 10 ng of cDNA in 96-well reaction plates on a StepOnePlus™ system (Applied Biosystems). All expression data were normalized by the $2^{(\Delta Ct)}$ method using 18S as internal control.

2.6. Western blot analysis

Muscle tissues and cell extracts were homogenized in a buffer containing 50 mM HEPES, pH 7.4, 2 mM EDTA, 150 mM NaCl, 30 mM $NaPPO_4$, 10 mM NaF, 1% Triton X-100, 1.5 mg/ml benzamidine HCl and 10 μl/ml of each: protease inhibitor, phosphatase I inhibitor, and phosphatase II inhibitor (Sigma–Aldrich). Tissue homogenates were centrifuged for 25 min at 15,000 *g*, and supernatants were stored at -80 °C. A total of 30 μg of solubilized proteins from muscle tissue and myotubes were run on a 4–12% SDS-PAGE (Bio-Rad), transferred onto nitrocellulose membrane (Hybond ECL, Amersham Biosciences), and blotted with the following primary antibodies: mG0S2 (#sc-133423, Santa Cruz Biotechnology Inc.), hG0S2 (#12091-1-AP, Protein Tech), ATGL (#2138, Cell Signaling Technology Inc.), and PDK4 (#H00005166-A02, Abnova). Subsequently, immunoreactive proteins were blotted with secondary HRP-coupled antibodies (Cell Signaling Technology Inc.) and revealed by enhanced chemiluminescence

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reagent (SuperSignal West Femto, Thermo Scientific), visualized using the ChemiDoc MP Imaging System, and data analyzed using the ImageLab 4.2 version software (Bio-Rad Laboratories, Hercules, USA). GAPDH (#2118, Cell Signaling Technology Inc.) was used as an internal control.

2.7. ATGL activity assay

ATGL activity was measured on muscle tissue and cell lysates (i.e. myotubes and COS7 cells stably overexpressing hATGL [24]) as previously described [25]. Briefly, tissue and cell lysates were extracted in a lysis buffer containing 0.25 M sucrose, 1 mM EDTA, 1 mM DTT, 20 μg/ml leupeptin, and 2 μg/ml antipain. [9, 10−^{3}H(N)] triolein (PerkinElmer), and cold triolein were emulsified with phospholipids by sonication. The emulsion was incubated for 30 min at 37 °C in the presence of 10−40 μg of total protein from tissue and cell lysates. After incubation, the reaction was terminated by adding 3.25 ml of methanol-chloroform-heptane (10:9:7) and 1.1 ml of 0.1 M potassium carbonate/0.1 M boric acid (pH 10.5). After centrifugation (800 *g*, 15 min), 0.5 ml of the upper phase was collected for scintillation counting. ATGL activity was measured in the presence of 1 μg of human recombinant G0S2 protein (rhG0S2) (OriGene), CGI-58 (rhCGI-58) (Abnova), or both. The data are expressed in nmol of oleic acid released per h per mg of protein.

2.8. Determination of glucose metabolism

Cells were pre-incubated with a glucose- and serum-free medium for 90 min, then exposed to DMEM supplemented with D[U-^{14}C] glucose (1 μCi/ml; PerkinElmer, Boston, MA). Following incubation, glucose oxidation was determined by counting of $^{14}CO_2$ released into the culture medium. The cells were then solubilized in KOH 30%, and glycogen synthesis was determined as previously described [26].

2.9. Determination of fatty acid metabolism

Cells were pulsed overnight for 18 h with [1−^{14}C] oleate (1 μCi/ml; PerkinElmer, Boston, MA) and cold oleate (100 μM) to prelabel the endogenous TAG pool. Oleate was coupled to FA-free BSA in a molar ratio of 5:1. Following the pulse, myotubes were chased for 3 h in DMEM containing 0.1 mM glucose, 0.5% FA-free BSA, and 10 μM triacsin C to block FA recycling into the TAG pool as described elsewhere [27]. TAG-derived FA oxidation was measured by the sum of $^{14}CO_2$ and ^{14}C-ASM (acid soluble metabolites) in the absence of triacsin C as previously described [22]. Myotubes were harvested in 0.2 ml SDS 0.1% at the end of the pulse and of the chase period to determine oleate incorporation into TAG, diacylglycerol (DAG), monoacylglycerol, FA, and protein content. The lipid extract was separated by TLC using heptane-isopropylether-acetic acid (60:40:4, v/v/v) as developing solvent. All assays were performed in duplicates, and data were normalized to cell protein content. Palmitate oxidation rate was measured as previously described [28].

2.10. Determination of mitochondrial content

We determined mitochondrial mass in myotubes using Mitotracker Green FM (Invitrogen, Carlsbad, CA), which stains mitochondrial matrix protein irrespective of the membrane potential and thus provides an accurate assessment of mitochondrial mass. Similarly, we measured mitochondrial membrane potential using a Mitotracker Red CMX-Ros (Invitrogen, Carlsbad, CA), which stains mitochondria according to their membrane potential. Briefly, cells were washed with PBS and incubated at 37 °C for 30 min with 100 nM of each Mitotracker. Cells were then harvested using trypsin/EDTA and resuspended in PBS. Fluorescence intensity was measured on a fluorometer and values expressed as relative fluorescence units (RFU).

2.11. Tissue-specific [2−^{3}H] deoxyglucose uptake *in vivo*

Muscle-specific glucose uptake was assessed in response to an intraperitoneal bolus injection of 2-[1,2−^{3}H(N)]deoxy-D-Glucose (PerkinElmer, Boston, Massachusetts) (0.4 μCi/g body weight) and insulin (3 mU/g body weight). The dose of insulin was determined in preliminary studies to reach a nearly maximal stimulation of insulin signaling and glucose uptake in all muscle types and metabolic tissues. Mice were fasted 2 h before injection, killed 30 min after injection, and tissues were extracted by precipitation of 2-deoxyglucose-6-phosphate as previously described [29].

2.12. Determination of triacylglycerol and ceramide content

Triacylglycerol and ceramide species content was determined by high-performance liquid chromatography-mass spectrometry after lipid extraction as described elsewhere [29,30].

2.13. Statistical analyses

All statistical analyses were performed using GraphPad Prism 5.0 for Windows (GraphPad Software Inc., San Diego, CA). Normal distribution and homogeneity of variance of the data were tested using Shapiro−Wilk and F tests, respectively. One-way ANOVA followed by Tukey's post hoc tests and Student's *t*-tests were performed to determine differences between treatments. Two-way ANOVA and Bonferroni's post hoc tests were used when appropriate. All values in figures and tables are presented as mean ± SEM. Statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. G0S2 is associated with lipid content and oxidative capacity in human skeletal muscle

Muscle G0S2 protein expression was assessed in human *vastus lateralis* samples in healthy lean, obese, and athlete volunteers. We observed that G0S2 protein content was increased in endurance-trained individuals compared to lean, while a trend was observed in obese sedentary volunteers (Figure 1A). Interestingly, muscle G0S2 protein content tightly correlates with ATGL protein ($r^2 = 0.67$, $p < 0.0001$) (Figure 1B). The ratio of G0S2 to ATGL protein content was not statistically different between lean, obese and athlete (data not shown). We also noted that G0S2 protein was positively associated with IMCL content ($r^2 = 0.17$, $p = 0.0048$) (Figure 1C), and cytochrome oxidase activity, a marker of muscle oxidative capacity ($r^2 = 0.20$, $p = 0.0021$) (Figure 1D). Together, these data indicate that G0S2 is significantly expressed in skeletal muscle and may play a role in the regulation of lipid storage and metabolism.

3.2. G0S2 inhibits ATGL activity in mouse and human skeletal muscle

Muscle G0S2 protein expression was measured in different types of mouse skeletal muscles. We observed that G0S2 protein content was higher in the oxidative *soleus* muscle compared to mixed *gastrocnemius* and glycolytic *extensor digitorum longus* muscles (Figure 2A). A similar expression pattern was observed for CGI-58 as previously described [14]. ATGL activity measured in lysates of both *soleus* and *EDL* muscles was significantly reduced by the addition of G0S2 recombinant protein (−44% and −47% respectively, $p < 0.05$) (Figure 2B). We observed a similar effect in lysates of human *vastus*

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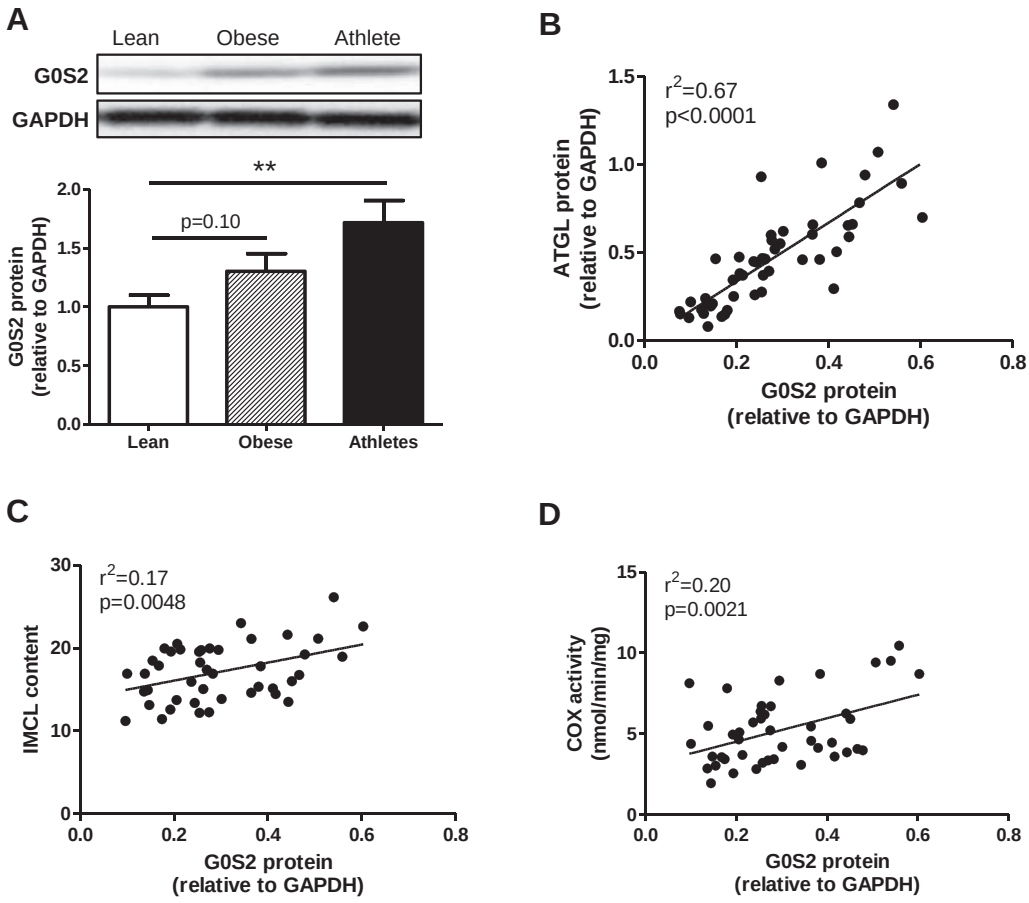

Figure 1: G0S2 is associated with lipid content and oxidative capacity in human skeletal muscle. (A) Representative blot and quantification of G0S2 protein content measured in *vastus lateralis* muscle of healthy lean, obese, and endurance-trained volunteers (n = 11 per group). Correlations between muscle G0S2 protein and ATGL protein (B), IMCL content (C), and cytochrome oxidase (COX) activity (D) (n = 50). **p < 0.01 versus lean.

lateralis muscle (−37%, p < 0.05) (Figure 2C). Interestingly, G0S2 strongly inhibited ATGL activity in COS-7 cell lysates stably overexpressing ATGL but also completely abolished its activation by the ATGL co-activator CGI-58 (Figure 2D). Collectively, these data suggest that G0S2 is a potent inhibitor of ATGL activity in mouse and human skeletal muscle.

3.3. G0S2 overexpression in human primary myotubes inhibits lipolysis and FA oxidation

G0S2 was overexpressed using an adenovirus containing G0S2 cDNA. An adenovirus containing GFP cDNA was used as a control. Adenovirus-mediated G0S2 overexpression led to a 4.3-fold increase of G0S2 gene expression (p < 0.001) (data not shown) and a 4.6-fold increase of protein content (p < 0.001) (Figure 3A) in 5 days differentiated human myotubes. Surprisingly, this was accompanied by a 21% increase of ATGL protein content in these cells (p < 0.001) (Figure 3B). As expected, ATGL activity was reduced by 61% in cells overexpressing G0S2 (p = 0.026) (Figure 3C).

We next examined the effect of G0S2 overexpression on lipolysis and FA metabolism. We observed that G0S2 overexpression increased TAG accumulation (+49%, p = 0.031) (Figure 3D), which was concomitantly accompanied by a decrease of FA release into the culture medium (−34%, p = 0.01) (Figure 3E) and FA oxidation (−38%, p = 0.038) (Figure 3F). Taken together, these data show that G0S2 inhibits ATGL activity in human primary myotubes, leading to a reduction of lipolysis rate and FA oxidation.

3.4. G0S2 knockdown in human primary myotubes promotes lipolysis and FA oxidation

G0S2 silencing in human primary skeletal muscle cells was realized using lentivirus containing an shRNA directed against G0S2 (shG0S2), and a scramble shRNA (shNT) was used as a control. No significant change in cell viability was observed between shNT and shG0S2 conditions (data not shown). Lentivirus-mediated G0S2 knockdown strongly reduced G0S2 gene expression (−61%, p = 0.009, data not shown), leading to a virtually complete loss of G0S2 protein (Figure 4A). Interestingly, G0S2 downregulation was accompanied by a significant decrease of ATGL protein (−39%, p = 0.026) (Figure 4B), despite a nearly 50% increase of ATGL activity (p = 0.027) (Figure 4C). G0S2 knockdown was associated with a marked reduction of the TAG pool (−68%, p < 0.001) (Figure 4D) that was accompanied by a robust increase of FA release (4.8 fold, p < 0.001) (Figure 4E) and FA oxidation (3 fold, p < 0.001) (Figure 4F). Collectively, these results show that G0S2 knockdown promotes lipolysis and increases FA oxidation in human primary myotubes.

3.5. G0S2 controls lipolysis in an ATGL-dependent manner in human primary myotubes

Because G0S2 may display effects on lipid metabolism independent of ATGL [31], we performed a double knockdown of G0S2 and ATGL and assessed lipid metabolism (Figure 5A). As previously observed, the TAG pool was strongly reduced in myotubes knocked down for G0S2, but this effect was totally abrogated and the TAG pool increased

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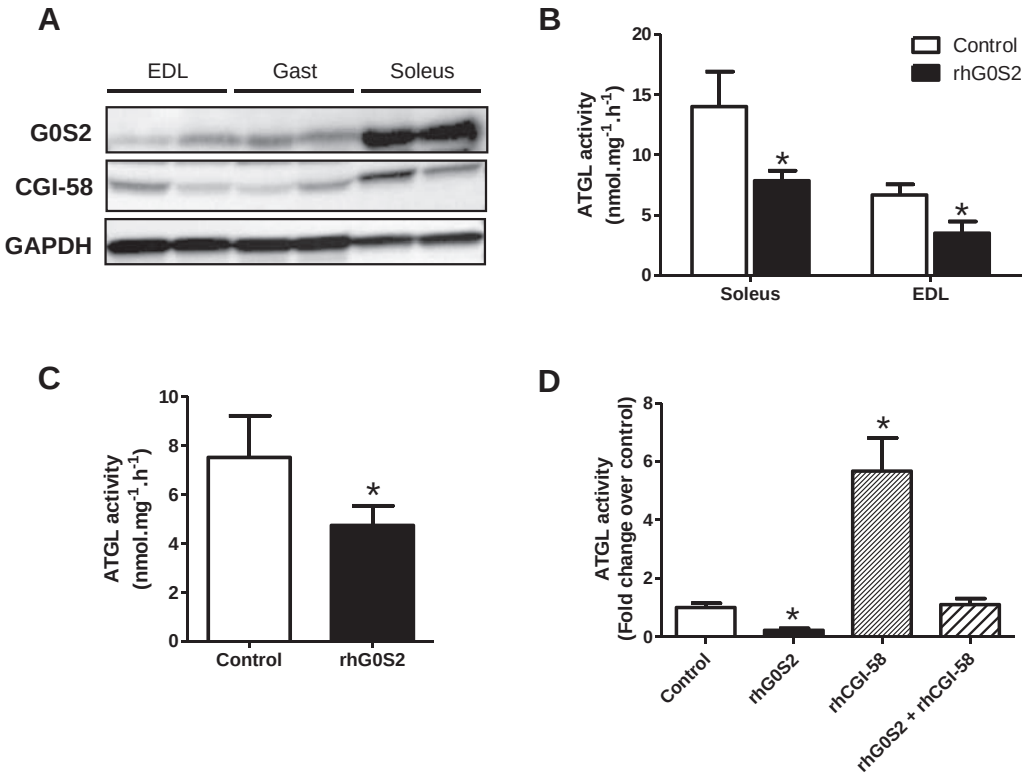

Figure 2: G0S2 inhibits ATGL activity in mouse and human skeletal muscle. (A) Representative blots of G0S2 and CGI-58 protein content in mouse *extensor digitorum longus* (EDL), *gastrocnemius* (Gast), and *soleus* skeletal muscles. Triacylglycerol hydrolase activity (TAGH) was measured in absence (control) or presence of recombinant human G0S2 (rhG0S2) in (B) mice *soleus* and *EDL* ($n = 7$) and (C) human *vastus lateralis* muscle ($n = 5$). (D) TAGH activity was measured in absence (control) or presence of recombinant human G0S2 (rhG0S2), CGI-58 (rhCGI-58), or both (rhG0S2+rhCGI-58) in COS7 cell extracts overexpressing human ATGL ($n = 4$). *$p < 0.05$ versus control.

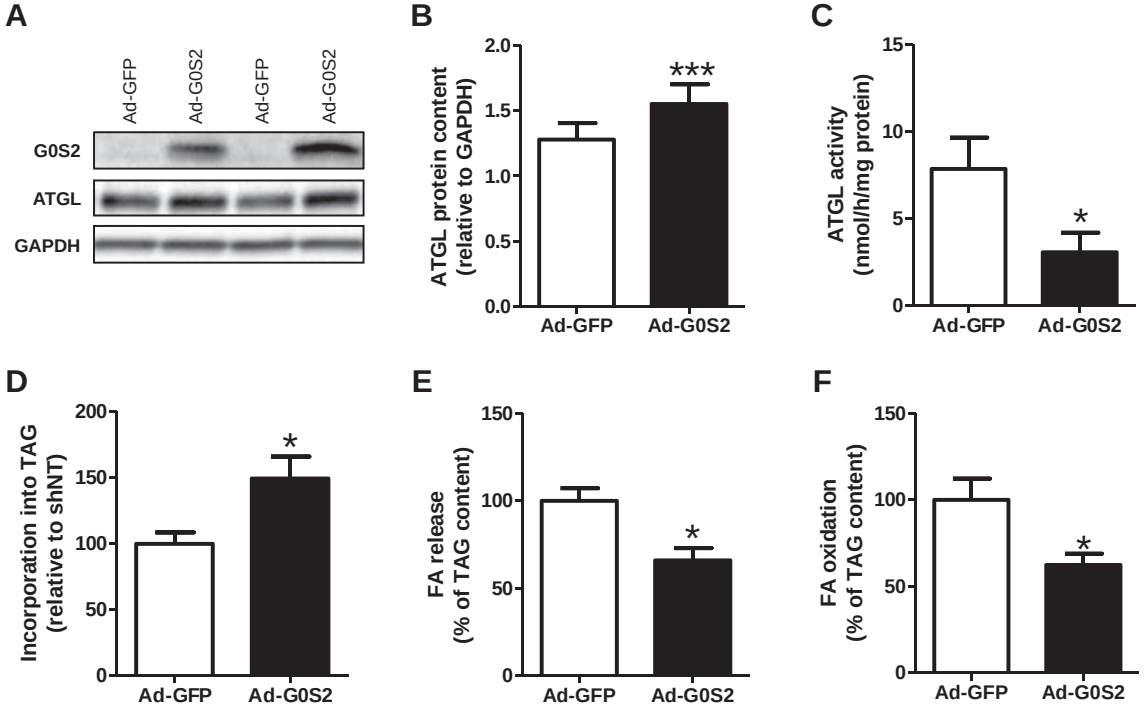

Figure 3: G0S2 overexpression in human primary myotubes inhibits lipolysis and FA oxidation. Representative blots (A) and quantification (B) of G0S2 and ATGL protein content measured in control myotubes (Ad-GFP) and myotubes overexpressing G0S2 (Ad-G0S2) ($n = 8$). (C) ATGL enzyme activity in control myotubes (Ad-GFP) and myotubes overexpressing G0S2 (Ad-G0S2) ($n = 3$). Pulse-Chase studies using [1−^{14}C] oleate were performed to determine the rate of (D) incorporation of radiolabeled oleate into TAG (Ad-GFP $= 41 \pm 11$ nmol/3 h/mg protein), (E) FA release (Ad-GFP $= 26.3 \pm 2.5$ nmol/3 h/mg protein), and (F) oleate oxidation (Ad-GFP $= 2.74 \pm 0.19$ nmol/3 h/mg protein) in control myotubes (Ad-GFP) and myotubes overexpressing G0S2 (Ad-G0S2) ($n = 6$). *$p < 0.05$, ***$p < 0.001$ versus Ad-GFP.

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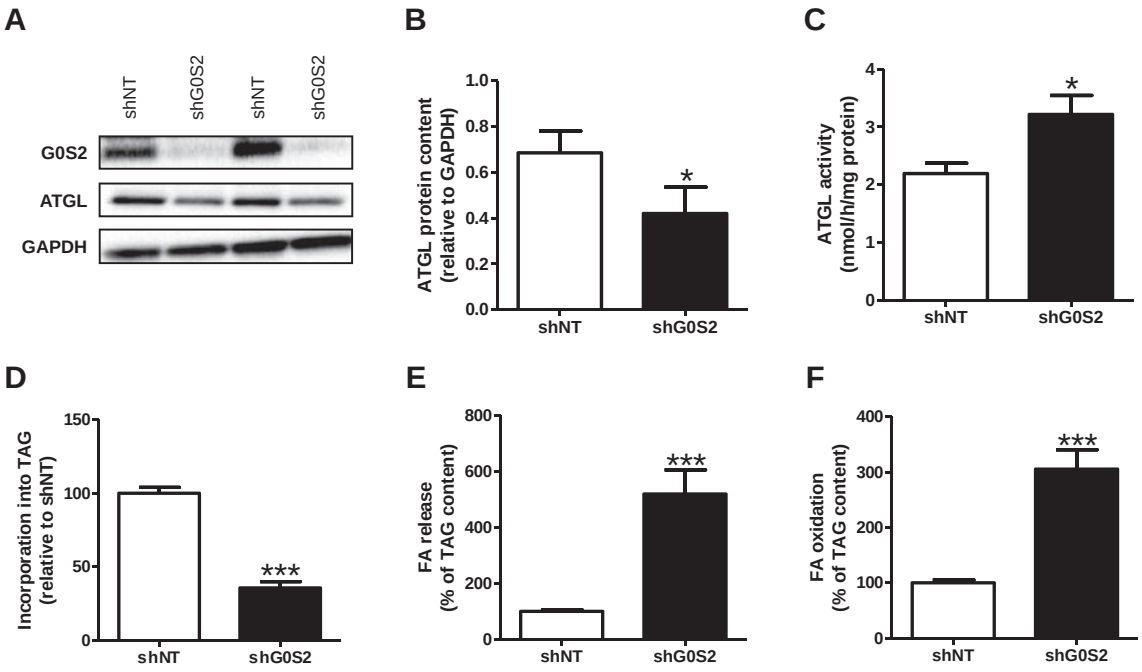

Figure 4: G0S2 knockdown in human primary myotubes promotes lipolysis and FA oxidation. Representative blots (A) and quantification (B) of G0S2 and ATGL protein content in control myotubes (shNT) and myotubes downregulated for G0S2 (shG0S2) ($n = 5$). (C) ATGL enzyme activity in control myotubes (shNT) and myotubes downregulated for G0S2 (shG0S2) ($n = 3$). Pulse-Chase studies using [1−^{14}C] oleate were performed to determine the rate of (D) incorporation of radiolabeled oleate into TAG (shNT = 41.8 ± 5.1 nmol/3 h/mg protein), (E) FA release (shNT = 74 ± 6 nmol/3 h/mg protein), and (F) oleate oxidation (shNT = 3.74 ± 0.32 nmol/3 h/mg protein) in control myotubes (shNT) and myotubes with G0S2 silencing (shG0S2). $n = 9$. *$p < 0.05$, ***$p < 0.001$ versus shNT.

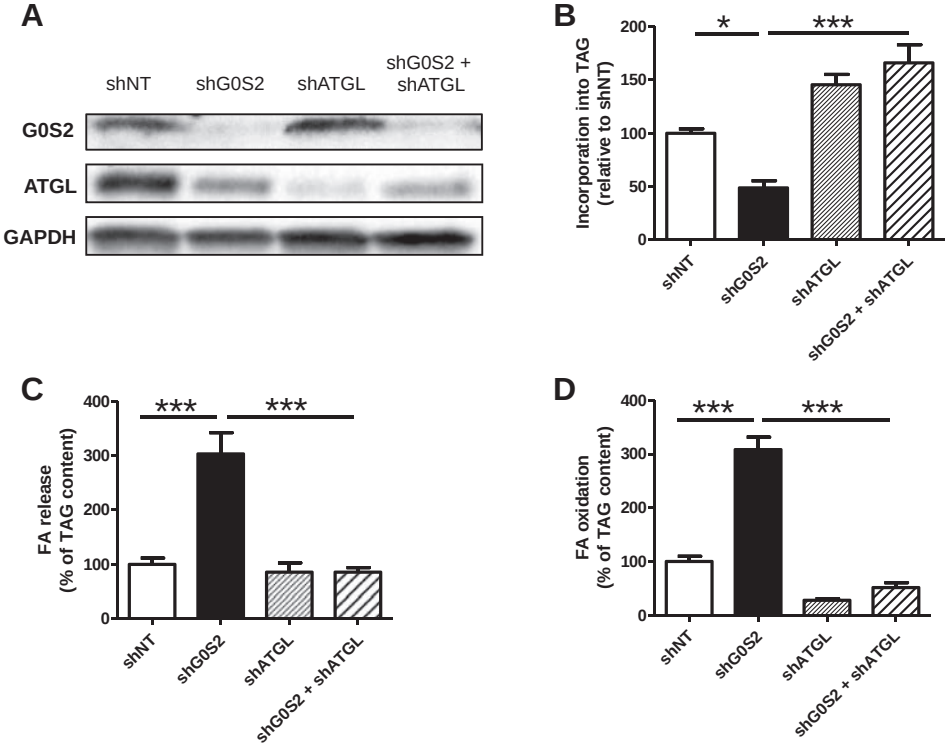

Figure 5: G0S2 controls lipolysis in an ATGL-dependent manner. (A) Representative blot of G0S2 and ATGL protein content in control myotubes (shNT), myotubes with a downregulation of G0S2 (shG0S2), ATGL (shATGL), or both (shG0S2+shATGL). Pulse-Chase studies using [1−^{14}C] oleate were performed to determine the rate of (B) incorporation of radiolabeled oleate into TAG (shNT = 40.2 ± 1.1 nmol/3 h/mg protein), (C) FA release (shNT = 66.3 ± 5.8 nmol/3 h/mg protein), and (D) oleate oxidation (shNT = 3.09 ± 0.13 nmol/3 h/mg protein) in these cells ($n = 3$). *$p < 0.05$, ***$p < 0.001$.

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(+65% *vs* shNT, p = 0.004) when ATGL was simultaneously ablated. A similar increase in TAG pool was observed when ATGL was down regulated alone (Figure 5B). In line with changes in TAG content, the increase of FA release (Figure 5C) and FA oxidation (Figure 5D) was totally blunted when both G0S2 and ATGL were knocked down, in a similar fashion as when ATGL was ablated alone. Altogether, these data suggest that G0S2 controls lipolysis and lipid metabolism in a strictly ATGL-dependent manner.

3.6. G0S2 knockdown reduces glucose metabolism and enhances mitochondrial function

FA and glucose are the main energy substrates in skeletal muscle, and a finely tuned regulation occurs for the use of these two substrates. Considering that G0S2 knockdown has an important impact on IMTG content and lipolysis, we investigated its effect on glucose metabolism. We observed that G0S2 knockdown decreased glucose oxidation (−34%, p = 0.003) (Figure 6A) and glycogen synthesis (−63%, p < 0.001) (Figure 6B). Because glucose oxidative metabolism is under the control of pyruvate dehydrogenase kinase 4 (PDK4), we measured expression of PDK4 gene, a well-known canonical target gene of Peroxisome Proliferator Associated Receptor β (PPARβ), a master regulator of muscle oxidative metabolism. Interestingly, we observed that PDK4 was highly induced by G0S2 knockdown (5.4 fold, p < 0.001), an effect that was strongly abolished when the cells were concomitantly treated with 500 nM of GSK0660, a specific antagonist of PPARβ (−70%, p < 0.001) (Figure 6C).

Because PPARγ coactivator 1α (PGC1α), another master regulator of oxidative metabolism is also a PPARβ-target gene, we measured its expression level and showed a 2-fold increase of PGC1α gene expression in myotubes knocked down for G0S2 (p = 0.019) (Figure 6D). This was consistently associated with an increase in mitochondrial mass (+67%, p < 0.001) (Figure 6E) and membrane potential (+108%, p = 0.043) (Figure 6F) in myotubes knocked down for G0S2. The membrane potential to mitochondrial mass ratio was unchanged, suggesting that the increase of mitochondrial membrane potential reflects the increase of total mitochondrial content, instead of a higher oxidative phosphorylation rate per mitochondrion (shNT 0.15 ± 0.02 *vs* shG0S2 0.19 ± 0.04, NS). We also noted a nearly significant increase of ATP synthase subunit alpha protein of complex V (p = 0.05) (data not shown). Altogether, these data show that G0S2 controls fuel selection (FA *vs* glucose) in skeletal muscle cells partly through the modulation of lipid ligand availability for PPARβ.

3.7. G0S2 knockdown *in vivo* increases lipolysis and induces ceramide accumulation in skeletal muscle

To assess the physiological role of G0S2 *in vivo*, we knocked down its expression by injecting an AAV1 containing an shRNA directed against G0S2 in *tibialis anterior* and *gastrocnemius* muscles into 14-week old C57BL/6J mice. Intramuscular AAV1-shRNA-G0S2 injection significantly reduced G0S2 mRNA and protein expression compared to the contralateral leg injected with an AAV1 containing a non-targeted shRNA (Supplemental Figure 1). Interestingly, and in line with *in vitro* data, knockdown of G0S2 tended to reduce ATGL protein content (−38%, p = 0.056) (Supplemental Figure 1) and decreased IMTG content (−28%, p = 0.047) (Figure 7A). This was accompanied by increased palmitate oxidation rate to CO2 (2.7-fold, p < 0.05), palmitate oxidation to ASM (acid soluble metabolites) (p = 0.052) and total oxidation tended to increase (1.4 fold, p < 0.05) (Figure 7B). Again, as observed *in vitro*, PDK4 protein was increased in G0S2 knocked down muscles (+23%, p = 0.03) (Figure 7C). Because an

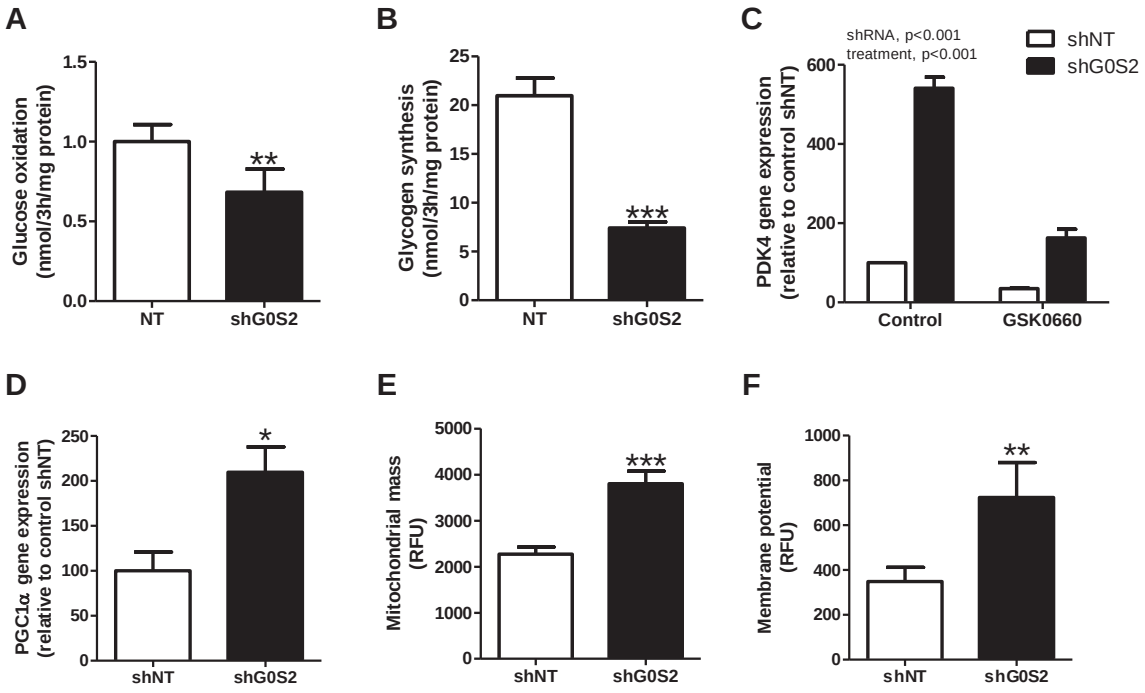

Figure 6: G0S2 knockdown reduces glucose metabolism and enhances mitochondrial function. (A) Glucose oxidation and (B) glycogen synthesis were measured in control myotubes (shNT) and myotubes knocked down for G0S2 (shG0S2) using [U−^{14}C] glucose. (C) PDK4 gene expression was measured in control myotubes (shNT) and myotubes knocked down for G0S2 (shG0S2) in absence or presence of a selective PPARδ antagonist GSK0660 500 nM PCG1α gene expression (D), mitochondrial mass (E), and mitochondrial membrane potential (F) were measured in control myotubes (shNT) and myotubes knocked down for G0S2 (shG0S2) (n = 9). *p < 0.05, **p < 0.01, ***p < 0.001 versus shNT.

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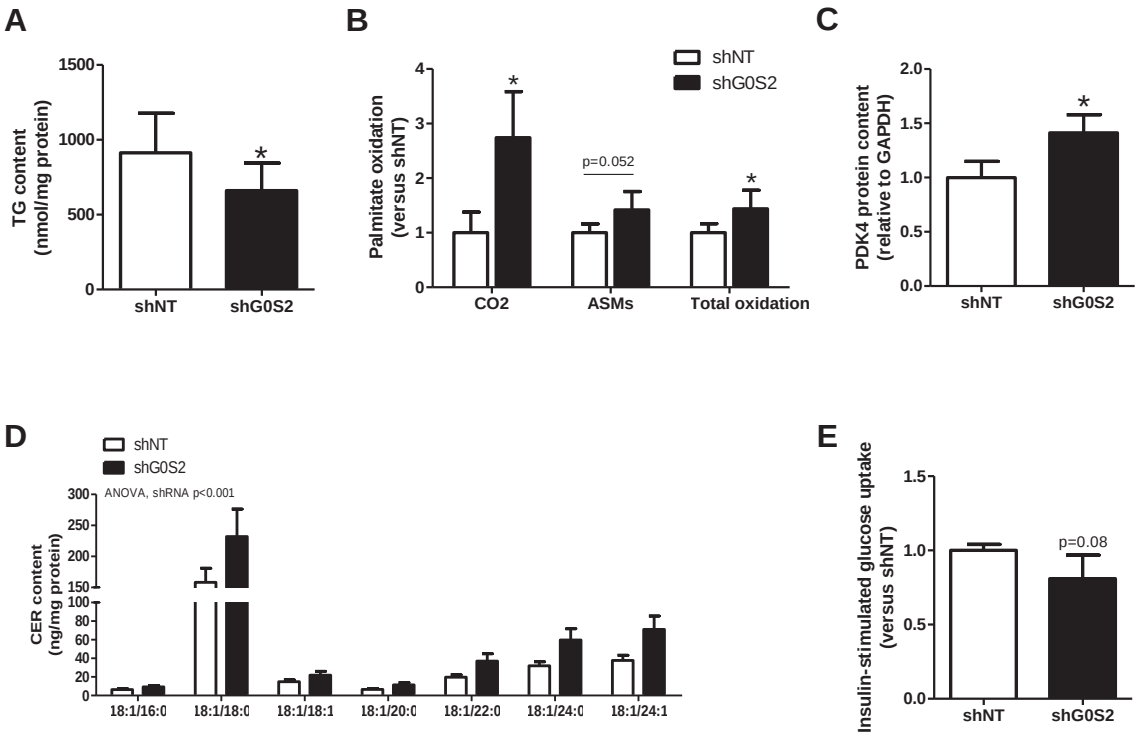

Figure 7: G0S2 knockdown increases FA oxidation and induces CER accumulation in skeletal muscle *in vivo*. (A) IMTG content was measured in control (shNT) and G0S2 silenced (shG0S2) mouse *tibialis anterior* homogenate (n = 7). (B) Palmitate oxidation rate was measured using [1−^{14}C] palmitate in control (shNT) and G0S2 silenced (shG0S2) muscle homogenates. Palmitate oxidation (i.e. CO2), acid soluble metabolites accumulation (i.e. ASMs) and total oxidation (i.e. the sum of CO2 release and ASMs accumulation, shNT = 965 ± 126 nmol/h/g tissue) were measured (n = 6). (C) PDK4 protein content was quantified by western blot (n = 6). (D) Ceramide (CER) subspecies content (n = 7), and (E) insulin-stimulated glucose uptake in control (shNT) and G0S2 knockdown (shG0S2) muscles (shNT = 13 ± 3 μmol/min/100 g tissue). (n = 6). *p < 0.05 versus shNT.

elevated rate of lipolysis in muscle may cause lipotoxicity and reduce insulin-mediated glucose uptake in a resting state, we next examined lipotoxic lipid species and glucose uptake. Interestingly, we observed a significant accumulation of different ceramide species (+60%) in muscles knocked down for G0S2 (Figure 7D), and this was associated with a non-significant trend for a lower insulin-stimulated glucose uptake (−19%, p = 0.08) (Figure 7E). We further observed a down-regulation of muscle G0S2 protein concomitant with reduced insulin-stimulated glucose uptake in HFD-fed mice with marked body weight gain (Supplemental Figure 2). Together, these *in vivo* data recapitulate what has been observed *in vitro* with G0S2 knockdown in myotubes, and strengthen the important metabolic role of G0S2 in skeletal muscle.

4. DISCUSSION

Obesity and type 2 diabetes are associated with an accumulation of ectopic lipids in insulin-sensitive "non-adipose" tissues such as skeletal muscle [4]. A growing body of data suggests that an altered handling of these lipids may be responsible for the impairment of insulin action [3]. As skeletal muscle acts as a metabolic sink for glucose in postprandial conditions [32,33], impaired insulin action in this organ has an important impact on whole-body glucose homeostasis. It is therefore important to understand how the IMTG pool is regulated. In this study, we identified G0S2 as an important regulator of lipid metabolism in mouse and human skeletal muscle. We showed that G0S2 is preferentially expressed in oxidative muscles, is associated with oxidative capacity and lipid content in human skeletal muscle, and inhibits ATGL activity. We further demonstrated that G0S2 plays a pivotal role in the regulation of intramyocellular lipolysis and fatty acid oxidation. Finally, we highlighted a potential role for G0S2 in providing lipolysis-derived fatty acids to activate PPARβ and downstream expression of its target genes to regulate energy metabolism in skeletal muscle.

We observed a higher expression of G0S2 in skeletal muscle of endurance-trained individuals compared to lean subjects. This is in line with a recent report showing that G0S2 protein is increased in rat skeletal muscle following 8 weeks of endurance training [34]. It is well known that athletes display high muscle oxidative capacity and IMCL content [8]. Interestingly, we found that G0S2 protein is associated with skeletal muscle oxidative capacity and IMCL levels. Of importance, we observed that G0S2 strongly correlates with ATGL protein content in human skeletal muscle, suggesting a role for G0S2 in the regulation of muscle lipid metabolism.

We observed a higher expression of G0S2 in an oxidative muscle *(soleus)* compared to more glycolytic muscles *(gastrocnemius* and *extensor digitorum longus)*, which goes along with a higher expression of CGI-58 and of all the proteins involved in the lipolytic machinery [35]. This is consistent with a high capacity to store, mobilize and oxidize lipids in oxidative muscles [36] and suggests that G0S2 may be essentially expressed in type I oxidative fibers, as observed for ATGL [37]. Studies from different groups report that G0S2 inhibits ATGL activity in adipose tissue [17,18] and in liver [19,20], and we show for the first time that G0S2 inhibits ATGL activity in human and mouse skeletal muscle. Mutagenesis studies have described that the hydrophobic domain of G0S2 can directly bind to the patatin-like domain of

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ATGL and that this interaction is required for the inhibitory action of G0S2 [17,38]. Interestingly, we observed in COS7 cells overexpressing ATGL that rhG0S2 is able to suppress ATGL activation induced by rhCGI-58. This suggests that G0S2 can bind ATGL independently of the presence of CGI-58, by a non-competing mechanism. However, the molecular mechanisms of ATGL inhibition by G0S2 are still poorly understood and clearly need to be further investigated.

To gain further insight into the functional role of G0S2 in skeletal muscle, G0S2 was downregulated in human primary myotubes. G0S2 silencing induced an increase of ATGL activity and lipolysis, reflected by a strong reduction of TAG content and concomitant increase of FA release. Surprisingly, this was also accompanied by a significant decrease of ATGL protein content. Conversely, G0S2 overexpression led to decreased lipolytic rate, associated with an increase of ATGL protein content. Taken together, these results suggest that a compensatory mechanism occurs when ATGL activity is modulated. Similar findings have been observed when CGI-58 was overexpressed and knocked-down in this model [14]. As no change in ATGL gene expression was observed, either in knockdown or overexpression studies (data not shown), we suggest that this regulation may take place at the post-transcriptional level, although the precise underlying mechanism still remains unknown. In agreement with the increased lipolytic flux in G0S2 silenced cells, we observed an increase in IMTG-derived fatty acid oxidation, the opposite being observed in G0S2 overexpressing cells. These results highlight a pivotal role for G0S2 in providing substrates for mitochondrial β-oxidation. Interestingly, we noted an increased mitochondrial mass and membrane potential in G0S2 silenced cells. However, the membrane potential to mitochondrial mass ratio remained unchanged, suggesting that the increase in membrane potential was due to the presence of a higher number and/or size of mitochondria. Consistently, the gene expression of *PGC1α*, a master regulator of oxidative metabolism and mitochondrial biogenesis, increased in these cells. A recent study reported that G0S2 positively regulates OXPHOS activity by directly interacting with ATP synthase [31]. However, double G0S2 and ATGL knockdown experiments demonstrated that the effects of G0S2 knockdown on TAG content, FA release, and oxidation were totally dependent of ATGL.

FA and glucose are the main energy sources in skeletal muscle, and it is now well established that a finely tuned regulation occurs for the use of these two substrates, the so called fuel selection [39]. We observed that, concomitantly with an increased lipolytic flux in G0S2 silenced cells, oxidative (i.e. glucose oxidation) and non-oxidative (i.e. glycogen synthesis) glucose metabolism was blunted. This was accompanied by a strong induction of the "switch-gene" *PDK4*. PDK4 is a mitochondrial protein inhibiting glucose oxidation in response to a high FA availability [40]. As PDK4 is a PPARβ-target gene [41], and considering that FA act as endogenous ligands of PPARβ [42], we hypothesized that IMTG-derived FA could activate PPARβ and induce PDK4 expression in G0S2 silenced cells [14]. Thus activation of lipolysis by G0S2 knockdown robustly induced PDK4 in a PPARβ-dependent manner.

Most importantly, we further observed that G0S2 knockdown in mouse *tibialis anterior* muscle reduced IMTG content, enhanced FA oxidation *in vivo*, and induced PDK4 expression. However, perhaps because of lower down regulation of G0S2 *in vivo* compared to *in vitro*, the magnitude of change in FA oxidation was moderate and no significant effect was observed on glucose oxidation (data not shown). Interestingly, we also observed an accumulation of various ceramide species, suggesting that an elevated rate of lipolysis in skeletal muscle can lead to lipotoxicity in the resting state despite a slightly elevated FA oxidation rate. A similar metabolic change is classically observed during high fat feeding in which upregulation of mitochondrial FA oxidation cannot prevent ceramide accumulation and insulin resistance [29,43,44]. In this study, G0S2 knockdown-mediated ceramide accumulation was associated with a non-significant decrease of insulin-stimulated glucose uptake as would be expected from the negative action of ceramide on insulin signaling [45]. Interestingly, data from mouse models highlight a tissue-specific role for G0S2 in the control of insulin sensitivity. Of importance, G0S2 knockout mice are more insulin-sensitive and glucose tolerant than wild type littermates when fed a high fat diet [46]. These mice are protected against liver steatosis, and liver-specific G0S2 deletion induces a similar phenotype as G0S2 global knockout mice [20]. On the other hand, G0S2 overexpression specifically in adipose tissue leads to an improvement of insulin and glucose tolerance and reduces circulating fatty acids level [47]. In line with these studies, we show that G0S2 knockdown in skeletal muscle causes a lipotoxic injury and reduces insulin action similarly to what is observed in skeletal muscle during high fat feeding. Thus the specific role of muscle G0S2 in the regulation of whole body insulin sensitivity and metabolism should be explored in muscle-specific G0S2 knockout mice.

5. CONCLUSION

Collectively, our data show for the first time that G0S2 inhibits ATGL activity in mouse and human skeletal muscle and plays a central role in regulating lipid metabolism and substrate oxidation. These results also suggest that changes in G0S2 expression may cause accumulation of lipotoxic species in skeletal muscle and subsequent impairment of insulin action. Future studies are needed to further elucidate the potential contribution of skeletal muscle G0S2 to insulin resistance, obesity, and type 2 diabetes.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2016.04.004.

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Figure S1. Validation of G0S2 knockdown in skeletal muscle *in vivo*. (A) G0S2 mRNA and (B) protein levels in control (shNT) and G0S2 knockdown (shG0S2) mouse *tibialis anterior* (n=4-7). (C) ATGL mRNA and (D) protein levels in control (shNT) and G0S2 knockdown (shG0S2) mouse *tibialis anterior* (n=7). *p<0.05, ** p<0.01 versus shNT.

Figure S2. Effect of high fat feeding on skeletal muscle G0S2 protein expression *in vivo*. (A) body weight, (B) insulin-stimulated glucose uptake and (C) G0S2 protein content in *tibialis anterior* muscle of mice fed normal chow diet (NCD) or high fat diet 60% fat (HFD) for 12 weeks (n=7). *p<0.05, *** p<0.001 versus NCD.

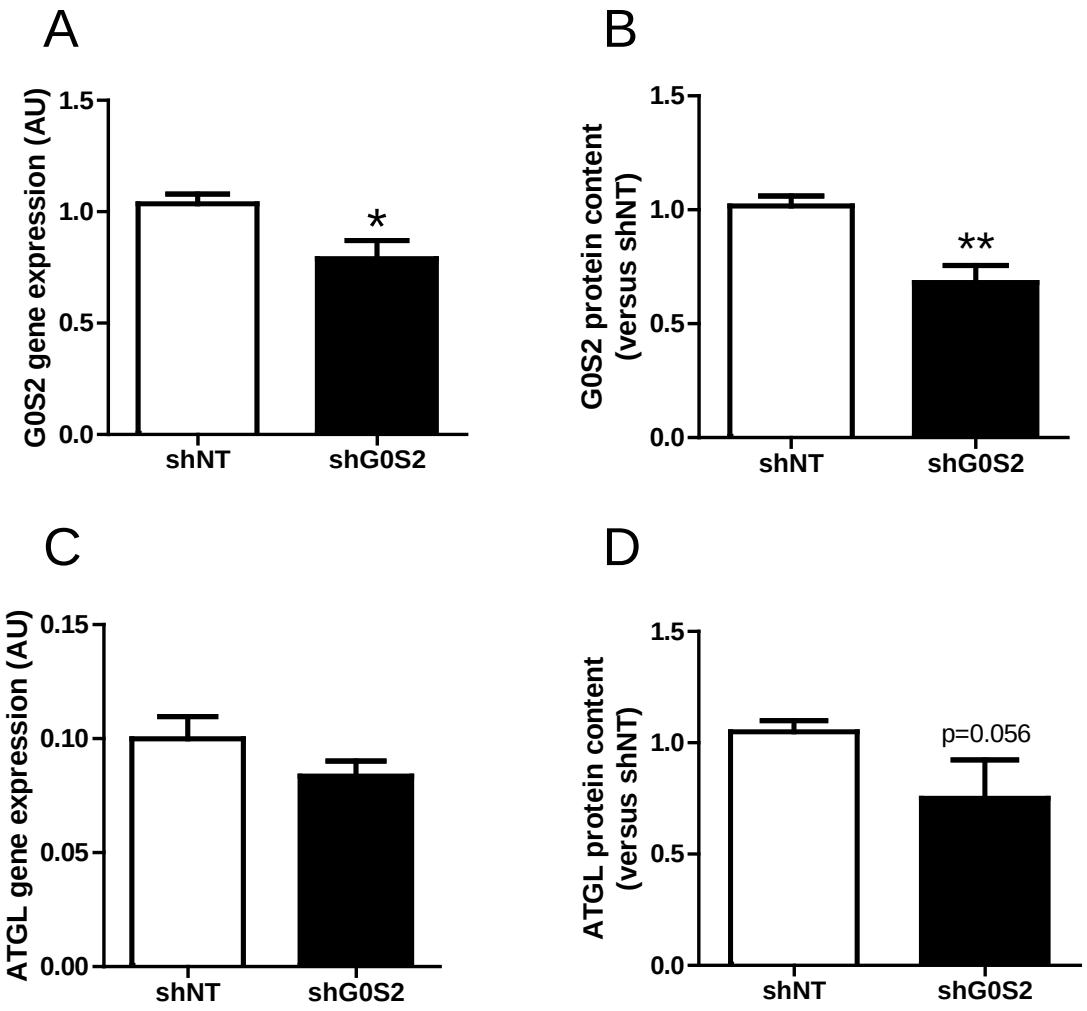
A
G0S2 gene expression (AU)
1.5
1.0
0.5
0.0
*
shNT
shG0S2
B
G0S2 protein content (versus shNT)
1.5
1.0
0.5
0.0
**
shNT
shG0S2
C
ATGL gene expression (AU)
0.15
0.10
0.05
0.00
shNT
shG0S2
D
ATGL protein content (versus shNT)
1.5
1.0
0.5
0.0
p=0.056
shNT
shG0S2

Figure S1

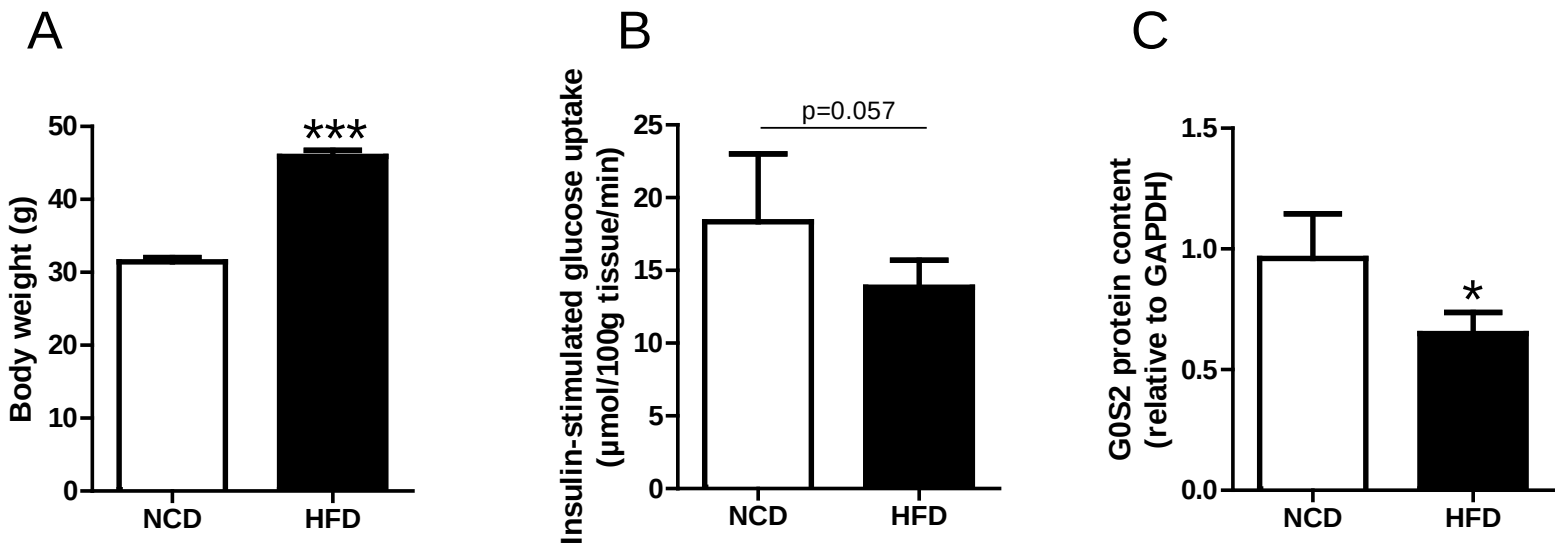
A
Body weight (g)
50
40
30
20
10
0
***
NCD
HFD
B
Insulin-stimulated glucose uptake
(µmol/100g tissue/min)
25
20
15
10
5
0
p=0.057
NCD
HFD
C
G0S2 protein content
(relative to GAPDH)
1.5
1.0
0.5
0.0
*
NCD
HFD

Figure S2

8.2.2. Discussion

Il est maintenant bien établi qu'une mauvaise gestion des lipides intramyocellulaires, et en particulier une dérégulation de la lipolyse, est impliquée dans l'altération de la sensibilité à l'insuline du muscle squelettique (Badin *et al.*, 2011). Cependant, la régulation de la lipolyse musculaire reste à ce jour mal comprise. ***Dans ce travail, nous nous sommes intéressés au rôle de la protéine G0S2 dans la régulation de l'ATGL, enzyme limitante de la lipolyse musculaire. Nous avons également évalué l'impact de modifications de l'expression de G0S2 sur le métabolisme lipidique et la sensibilité à l'insuline du muscle squelettique.***

Nous avons dans un premier temps montré que G0S2 est plus fortement exprimé dans un muscle oxydatif que dans un muscle glycolytique chez la souris. Chez l'homme, G0S2 est davantage exprimé dans le muscle d'individus entraînés en endurance par rapport à des sujets sédentaires, et est positivement corrélé à la capacité oxydative musculaire. Des études de différents groupes de recherche ont mis en évidence que G0S2 est capable d'inhiber l'activité de l'ATGL dans le tissu adipeux (Schweiger *et al.*, 2012; Yang *et al.*, 2010) et le foie (Wang *et al.*, 2013; Zhang *et al.*, 2014). De façon intéressante, nous avons constaté que l'expression musculaire de G0S2 est associée à celle de l'ATGL, et nous avons montré pour la première fois que G0S2 est capable d'inhiber l'activité de l'ATGL dans le muscle squelettique humain et murin. De façon surprenante, nous avons démontré que G0S2 est capable d'inhiber l'activité de l'ATGL, y compris lorsque cette dernière est activée par CGI-58 (figure 19). Ces résultats indiquent que G0S2 semble capable de lier l'ATGL indépendamment de la présence de CGI-58, par un mécanisme non-compétitif.

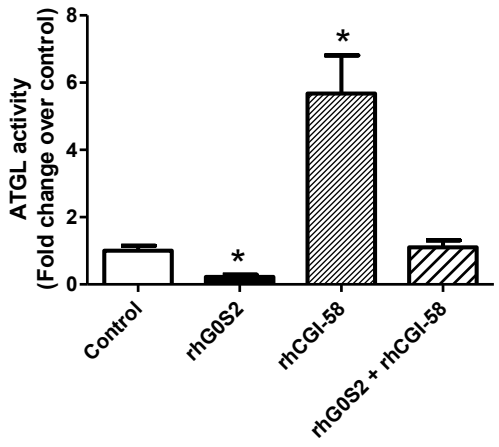

Figure 19. Mesure de l'activité de l'ATGL en présence de CGI-58 et G0S2

Mesure de l'activité triacylglycérol hydrolase dans des lysats de cellules COS7 surexprimant l'ATGL humaine, en absence (control) ou en présence de protéine recombinante humaine G0S2 (rhG0S2), CGI-58 (rhCGI-58), ou les deux (rhG0S2+rhCGI-58). n=4. *p<0.05 vs control.

Dans un deuxième temps, afin d'étudier le rôle fonctionnel de G0S2 dans le muscle, nous avons modulé son expression génique dans des cultures primaires de cellules musculaires humaines. Nous avons notamment mis en évidence qu'une invalidation de G0S2 dans ces cellules entraîne une augmentation de l'activité de l'ATGL. Ceci est associé à une diminution du contenu en IMTG et une augmentation de la libération d'AG dans le milieu, reflétant une activation de la lipolyse musculaire, de façon strictement dépendante de l'ATGL. En parallèle, nous avons constaté que l'oxydation des AG est augmentée dans les cellules invalidées pour G0S2, probablement via un effet de flux dû à la levée du frein exercé par G0S2 sur l'activité de l'ATGL permettant l'activation de la lipolyse et la libération d'AG oxydables par la mitochondrie, et que ceci s'accompagne d'une diminution de l'oxydation du glucose dans ces cellules (figure 20).

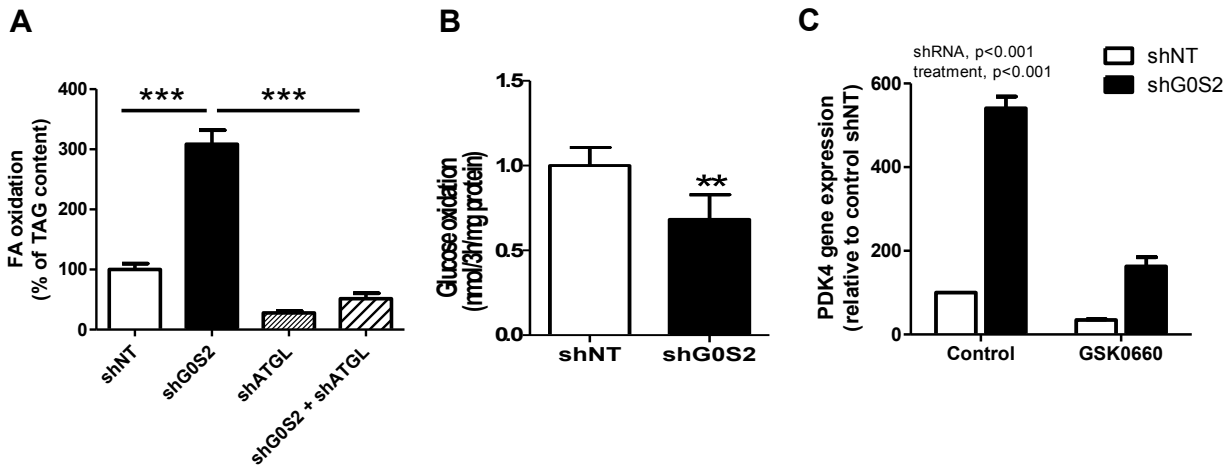

Figure 20. Contrôle de l'utilisation des substrats énergétiques par G0S2

(A) Mesure de l'oxydation d'AG radiomarqués dans des myotubes contrôle (shNT) ou invalidés pour G0S2 (shG0S2), l'ATGL (shATGL) ou les deux (shG0S2+shATGL) (n=3). (B) Mesure de l'oxydation de glucose radiomarqué dans des myotubes contrôle (shNT) ou invalidés pour G0S2 (shG0S2). (C) Expression génique de PDK4 mesurée dans des myotubes contrôle (shNT) ou invalidés pour G0S2 (shG0S2) en absence (control) ou en présence (GSK0660) d'un antagoniste spécifique de PPARβ (GSK0660 à 500nM). n=9. **p<0.01, ***p<0.001

Ces résultats sont en accord avec la régulation fine contrôlant l'utilisation des substrats énergétiques au sein du muscle squelettique (Randle *et al.*, 1963). Plusieurs mécanismes ont été décrits pour expliquer ce switch dans l'utilisation des AG et du glucose, parmi lesquels la modulation de l'activité de la *pyruvate dehydrogenase kinase 4* (PDK4), enzyme inhibant l'oxydation mitochondriale du glucose (Spriet *et al.*, 2004). En accord avec les résultats décrits précédemment,

nous avons observé dans les cellules invalidées pour G0S2 une augmentation de l'expression génique de PDK4. De façon intéressante, l'augmentation de PDK4 induite par l'invalidation de G0S2 est fortement diminuée en présence d'un inhibiteur spécifique de PPARβ (figure 20). Etant donné que PDK4 est un gène cible de PPARβ (Ehrenborg and Krook, 2009), et qu'il a été décrit que les AG issus de la lipolyse sont capables d'activer ce facteur de transcription (Bindesboll *et al.*, 2013), nous avons émis l'hypothèse que l'augmentation du flux lipolytique dans les cellules invalidées pour G0S2 fournit des ligands endogènes de PPARβ, entraînant une augmentation de l'expression de PDK4 et une diminution de l'oxydation du glucose (figure 21). En accord avec ces résultats, nous avons également noté une augmentation de *PPAR Gamma Coactivator 1α* (PGC1α) dans ces cellules, gène clé du métabolisme oxydatif musculaire et également cible de PPARβ.

Enfin, nous avons évalué l'impact d'une invalidation de G0S2 *in vivo* dans le muscle squelettique chez la souris. En accord avec les résultats obtenus dans les cultures primaires de cellules musculaires humaines, nous observons une diminution du contenu en IMTG associée à une augmentation de l'oxydation des AG et une induction de PDK4 dans les muscles invalidés pour G0S2. Cependant, l'amplitude de ces effets est plus modérée que dans les cultures cellulaires, probablement car l'extinction de G0S2 obtenue *in vivo* est plus faible que celle obtenue *in vitro*. Cependant, malgré une diminution modeste de G0S2 dans le muscle squelettique de souris, nous avons pu mettre en évidence une tendance à la diminution du transport de glucose stimulé par l'insuline dans les muscles invalidés pour G0S2, associée à une augmentation du contenu intracellulaire de céramides. Il semble donc que l'augmentation du flux lipolytique induite par l'invalidation de G0S2 *in vivo* entraîne une lipotoxicité conduisant à une altération de la sensibilité à l'insuline, malgré une légère augmentation de l'oxydation des AG. Il est important de souligner que des phénomènes similaires sont observés lors du développement d'une insulino-résistance induite par un régime riche en graisses, contexte dans lequel l'augmentation de l'oxydation des AG ne suffit pas à prévenir d'une accumulation de céramides (Badin *et al.*, 2013; Hancock *et al.*, 2008; Oakes *et al.*, 2013). Il est toutefois important de noter qu'un rôle tissu-spécifique de G0S2 sur la sensibilité à l'insuline semble se dégager des études réalisées dans des modèles animaux. Il a en effet été montré que les souris dans lesquelles G0S2 a été délété sont

partiellement protégées de l'insulino-résistance induite par un régime hyperlipidique, ainsi que du développement d'une stéatose hépatique. De façon intéressante, un phénotype similaire est retrouvé dans des souris délétées pour G0S2 spécifiquement dans le foie (El-Assaad *et al.*, 2015; Zhang *et al.*, 2014). D'autre part, la surexpression de G0S2 dans le tissu adipeux induit une amélioration de la tolérance à l'insuline et au glucose et diminue les niveaux d'AG circulants (Heckmann *et al.*, 2014). Nous montrons dans cet article qu'une invalidation de G0S2 dans le muscle génère une pression lipotoxique associée à une diminution de l'action de l'insuline, de façon similaire à ce qui est observé lors d'un régime gras. Il sera important dans de futurs travaux d'étudier l'impact d'une délétion musculaire de G0S2 dans la sensibilité à l'insuline systémique.

En résumé, ce travail démontre pour la première fois que G0S2 est un inhibiteur de l'activité de l'ATGL dans le muscle squelettique, et joue un rôle central dans la régulation du métabolisme lipidique et de l'oxydation des substrats énergétiques au sein de cet organe (Figure 21). De plus, nos résultats montrent que des dérégulations de l'expression musculaire de G0S2 pourraient contribuer à une altération de la sensibilité à l'insuline. Des études complémentaires seront nécessaires pour évaluer le rôle spécifique de G0S2 dans le développement de l'insulino-résistance musculaire, dans un contexte d'obésité et de diabète de type 2.

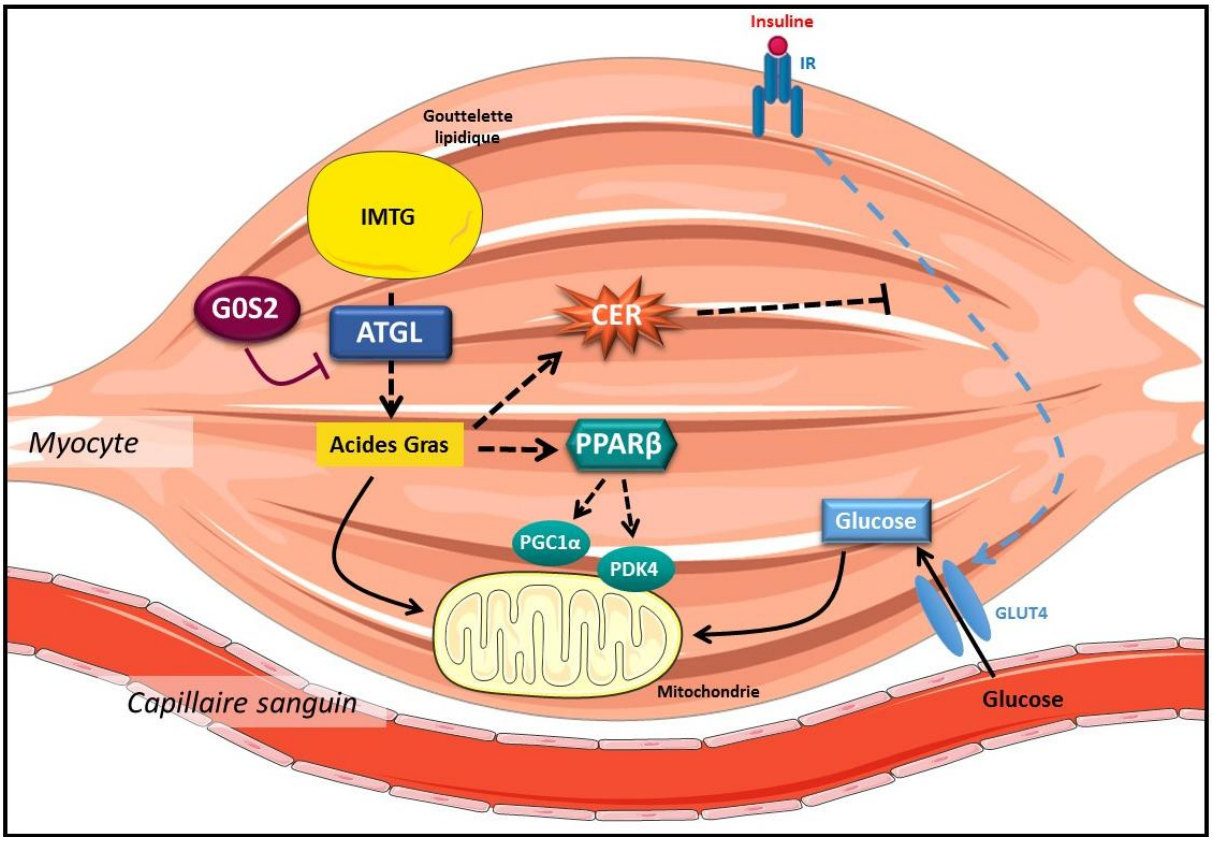

Figure 21. Schéma représentant le rôle de G0S2 dans le muscle squelettique

Dans le muscle squelettique, *G_0/G_1 Switch Gene 2* (G0S2) inhibe l'activité de l'*adipose triglyceride lipase* (ATGL). Une diminution de l'expression de G0S2 entraîne une augmentation de l'accumulation d'acides gras pouvant activer le *peroxysome proliferator-activated receptor β* (PPARβ) d'une part, mais également entraîner l'accumulation intramyocellulaire de céramides (CER). IMTG : triacylglycérols intramyocellulaires ; IR : récepteur à l'insuline ; GLUT4 : *glucose transporter 4* ; PDK4 : pyruvate déshydrogénase kinase 4 ; PGC1α : *peroxysome proliferator-activated receptor gamma coactivator 1α*

8.3. PUBLICATION 3 : LA PERILIPINE 5 AJUSTE L'OXYDATION DES LIPIDES AUX BESOINS METABOLIQUES ET PROTEGE D'UNE LIPOTOXICITE DANS LE MUSCLE SQUELETTIQUE

PLIN5 est une protéine présente à la surface des gouttelettes lipidiques appartenant à la famille des périlipines, identifiée en 2006 par le groupe de P.E. Bickel et décrite comme fortement exprimée dans les tissus oxydatifs tels que le foie, le cœur et le muscle squelettique (Wolins *et al.*, 2006b). Il a par la suite été mis en évidence que PLIN5 est capable d'interagir avec l'ATGL et CGI-58 (Granneman *et al.*, 2011; MacPherson *et al.*, 2013; Mason and Watt, 2015), et est un gène cible de PPARβ, acteur clé du métabolisme oxydatif musculaire (Bindesboll *et al.*, 2013). De façon intéressante, alors que PLIN5 semble jouer un rôle de barrière lipolytique en conditions basales dans le muscle squelettique chez la souris (Bosma *et al.*, 2013), il a été suggéré que cette protéine permettrait de faciliter la lipolyse lorsque les besoins énergétiques augmentent, par exemple lors de la pratique d'un exercice physique (Shepherd *et al.*, 2013). La relation entre PLIN5 et le métabolisme oxydatif d'une part, et la sensibilité à l'insuline d'autre part n'est toutefois pas évidente à ce jour. En effet, il a été montré qu'une surexpression musculaire de PLIN5 chez la souris n'est pas associée à des modulations de la sensibilité à l'insuline, mais s'accompagne d'une augmentation d'un cluster de gènes impliqués dans le métabolisme oxydatif (Bosma *et al.*, 2013). De façon contradictoire, une surexpression cardiaque (Pollak *et al.*, 2013) ou hépatique (Trevino *et al.*, 2015) de PLIN5 diminue l'expression de gènes impliqués dans l'oxydation mitochondriale, et est associée à une amélioration de la sensibilité à l'insuline (Trevino *et al.*, 2015). De plus, des animaux dont le gène codant pour PLIN5 a été délété ne présentent soit pas de modifications (Wang *et al.*, 2015) soit une amélioration (Mason *et al.*, 2014) de la sensibilité à l'insuline systémique.

Nous avons donc cherché à caractériser le rôle spécifique de PLIN5 dans le muscle squelettique sur le contrôle du flux lipolytique et du métabolisme oxydatif, ainsi que sur la lipotoxicité et la sensibilité à l'insuline musculaire. Le rôle fonctionnel de PLIN5 a été évalué non seulement en conditions basales, mais également lors d'une augmentation de la demande métabolique induite soit par une stimulation β-adrénergique, soit par la contraction des cellules musculaires. Pour cela, nous avons

développé au laboratoire un modèle de contraction de cellules musculaires humaines *in vitro*, qui sera présenté dans une partie méthodologique précédant l'article. Enfin, nous nous sommes intéressés au rôle de PLIN5 dans le muscle squelettique *in vivo* chez la souris, en conditions standard et suite à un challenge métabolique induit par la prise d'un régime riche en graisses, et nous avons évalué la sensibilité musculaire à l'insuline de ces animaux.

8.3.1. Caractérisation du modèle de contraction de cellules musculaires humaines in vitro

Nous disposons au laboratoire d'un système permettant de stimuler électriquement les cellules afin d'induire leur contraction (EPS, *Electrical Pulse Stimulation*). Nous avons mis au point deux protocoles de stimulation, permettant de mimer *in vitro* un exercice physique de courte durée et forte intensité d'une part (*i.e.* modèle de stimulation « aigue », exercice intense), et un exercice de longue durée et faible intensité d'autre part (*i.e.* modèle de stimulation « chronique », exercice modéré). Les protocoles utilisés pour mesurer les différents paramètres présentés ci-dessous (*i.e.* oxydation du glucose et des AG, synthèse de glycogène, expression génique) sont détaillés dans la section « matériels et méthodes » de l'article.

8.3.1.1. Caractérisation du modèle d'exercice intense

Les cellules musculaires humaines en culture primaire sont stimulées pendant 3h par des pulsations électriques de forte intensité à une fréquence élevée (10V, 24ms, 0.5Hz). Nous observons que les réserves de glycogène sont fortement déplétées dans les cellules ayant subi les stimulations électriques (Figure 22A), ce qui est associé à une augmentation de la libération de lactate (Figure 22B). En revanche, nous n'observons pas de modification de l'oxydation du glucose dans ces cellules (Figure 22C), mais une diminution de l'oxydation du palmitate (Figure 22D). L'expression génique de PGC1α, acteur clé du métabolisme oxydatif musculaire, n'est pas modifiée dans les cellules ayant subi les stimulations électriques (Figure 22E) mais, de façon intéressante, nous observons une augmentation de l'expression génique de certaines myokines (*i.e.* sécrétions musculaires de nature protéique) (Figure 22F).

Collectivement, ces résultats semblent indiquer que lorsque les cellules musculaires sont soumises à des stimulations de forte intensité, elles utilisent leurs réserves de glycogène pour produire de l'énergie, via la voie de la glycolyse anaérobie, entraînant ainsi une forte production de lactate, comme observé chez l'homme lors de la pratique d'un exercice court et intense. Ces cellules ne semblent

pas utiliser les AG comme source d'énergie dans des conditions de stimulation mimant un exercice intense de courte durée (Figure 18).

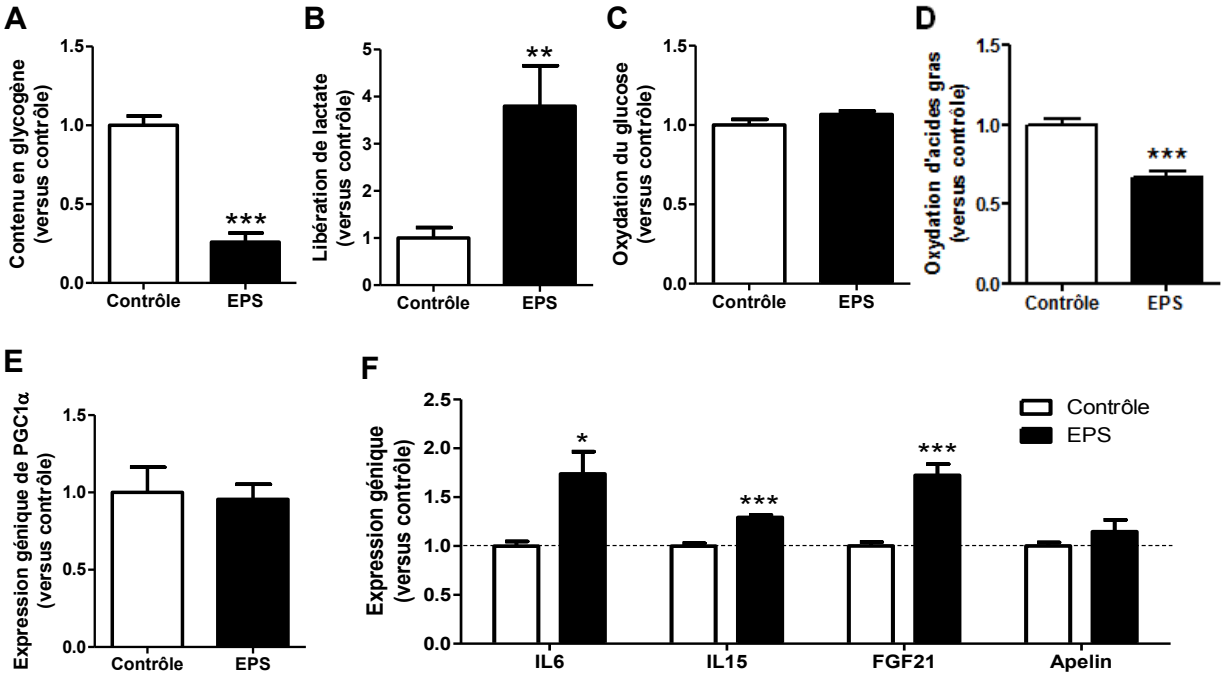

Figure 22. Caractérisation du modèle d'exercice intense *in vitro*

(A) Contenu en glycogène total (n=12), (B) libération de lactate (n=12), (C) oxydation du glucose et (D) des acides gras (n=6) dans des myotubes contrôle ou stimulés électriquement pendant 3h (EPS). Expression génique de (E) PGC1α (n=4) et (F) de myokines dans des myotubes contrôle ou EPS (n=8). *p<0.05, **p<0.01, ***p<0.001 versus contrôle

8.3.1.2. Caractérisation du modèle d'exercice modéré

Les cellules musculaires humaines en culture primaire sont stimulées pendant 24h par des pulsations électriques de faible intensité à une fréquence relativement faible (10V, 2ms, 0.1Hz). Dans ces conditions de stimulation, nous n'observons pas de diminution significative du contenu en glycogène dans les cellules stimulées (Figure 23A). Ceci peut être expliqué par une augmentation de la capacité de synthèse de glycogène mesurée dans ces cellules (Figure 23B), qui représente une adaptation classique du muscle squelettique observée lors d'un entraînement en endurance chez l'homme. De façon intéressante, nous observons une forte augmentation de l'oxydation du glucose (Figure 23C) et du palmitate (Figure 23D) dans les cellules stimulées électriquement pendant 24h. De plus, en parallèle de cette augmentation du métabolisme oxydatif, nous constatons une augmentation de

l'expression génique de PGC1α dans ces cellules (Figure 23E). Enfin, l'expression génique de certaines myokines est induite lors de la stimulation à faible intensité des cellules, mais de façon différente aux myokines induites par les stimulations de forte intensité (Figure 23F).

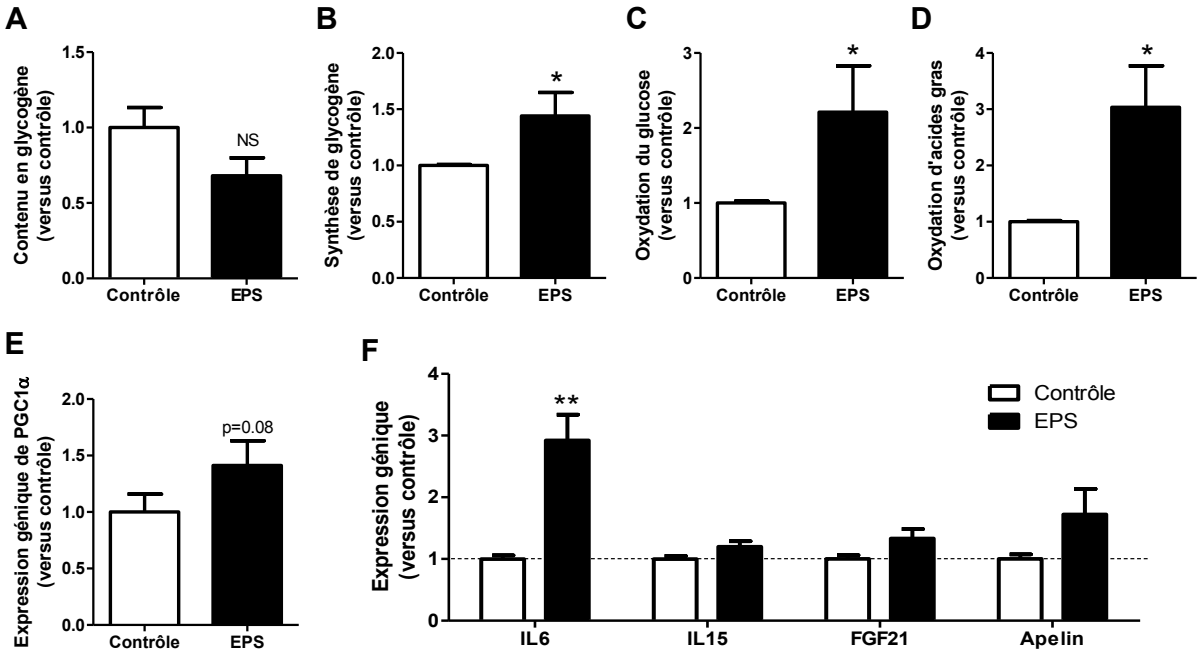

Figure 23. Caractérisation du modèle d'exercice modéré *in vitro*

(A) Contenu en glycogène total (n=4), (B) synthèse de glycogène (n=8), (C) oxydation du glucose (n=8) et (D) des acides gras (n=4) dans des myotubes contrôle ou stimulés électriquement pendant 24h (EPS). Expression génique de (E) PGC1α (n=4) et (F) de myokines dans des myotubes contrôle ou EPS (n=8). *p<0.05, **p<0.01, ***p<0.001 versus contrôle

L'ensemble de ces résultats semble indiquer que lorsque les cellules musculaires sont soumises à des stimulations de faible intensité de façon prolongée, elles utilisent préférentiellement des mécanismes en aérobie pour produire de l'énergie, via l'oxydation mitochondriale du glucose et des AG, mimant ainsi ce qui est observé chez l'homme lors de la pratique d'un exercice d'endurance (Figure 24). Nous avons donc choisi d'utiliser ce protocole de stimulation mimant un exercice d'intensité modérée induisant une augmentation de l'oxydation des substrats énergétiques pour étudier le rôle de PLIN5 sur le métabolisme oxydatif musculaire lorsque les besoins en énergie des cellules sont accrus.

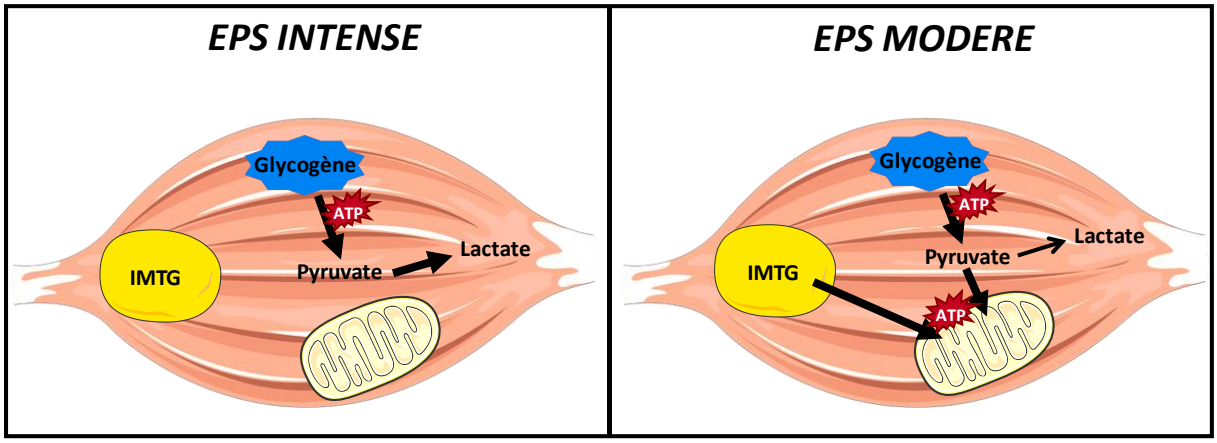

Figure 24. Modèle représentant l'utilisation des substrats énergétiques dans les cellules stimulées électriquement *in vitro*

Dans le modèle d'exercice intense, les cellules produisent de l'énergie principalement de façon anaérobie, via la glycolyse, conduisant à la production de lactate. Dans le modèle d'exercice modéré, les cellules musculaires oxydent des acides gras issus des triacylglycérols intramyocellulaires (IMTG) et du glucose pour produire de l'énergie. ATP : adénosine triphosphate

8.3.2. Publication 3

Perilipin 5 fine-tunes lipid oxidation to metabolic demand

and protects against lipotoxicity in skeletal muscle

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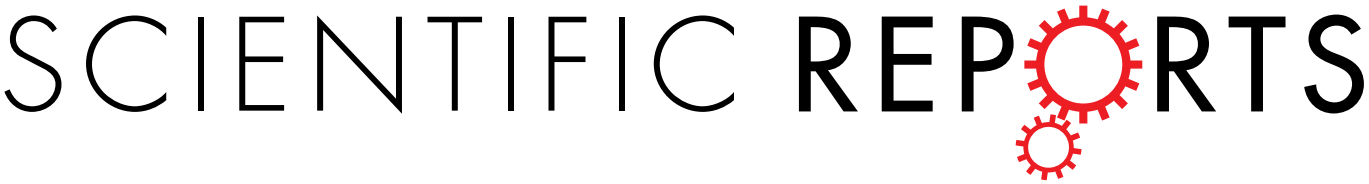

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Perilipin 5 fine-tunes lipid oxidation to metabolic demand and protects against lipotoxicity in skeletal muscle

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Lipid droplets (LD) play a central role in lipid homeostasis by controlling transient fatty acid (FA) storage and release from triacylglycerols stores, while preventing high levels of cellular toxic lipids. This crucial function in oxidative tissues is altered in obesity and type 2 diabetes. Perilipin 5 (PLIN5) is a LD protein whose mechanistic and causal link with lipotoxicity and insulin resistance has raised controversies. We investigated here the physiological role of PLIN5 in skeletal muscle upon various metabolic challenges. We show that PLIN5 protein is elevated in endurance-trained (ET) subjects and correlates with muscle oxidative capacity and whole-body insulin sensitivity. When overexpressed in human skeletal muscle cells to recapitulate the ET phenotype, PLIN5 diminishes lipolysis and FA oxidation under basal condition, but paradoxically enhances FA oxidation during forskolin- and contraction- mediated lipolysis. Moreover, PLIN5 partly protects muscle cells against lipid-induced lipotoxicity. In addition, we demonstrate that down-regulation of PLIN5 in skeletal muscle inhibits insulin-mediated glucose uptake under normal chow feeding condition, while paradoxically improving insulin sensitivity upon high-fat feeding. These data highlight a key role of PLIN5 in LD function, first by finely adjusting LD FA supply to mitochondrial oxidation, and second acting as a protective factor against lipotoxicity in skeletal muscle.

Cytosolic lipid droplets (LD) are important energy-storage organelles in most tissues[1]. LD are composed of a lipid core, mainly made of triacylglycerols (TAG), surrounded by a phospholipid monolayer in which are embedded proteins[2,3]. LD are dynamic organelles playing a central role in fatty acid (FA) trafficking[4]. Importantly, it has been suggested that altered LD dynamics could contribute to the development of muscle insulin resistance, by facilitating the emergence of cellular toxic lipids such as diacylglycerols (DAG) and ceramides (CER) known to impair insulin action[5,6]. LD therefore buffers intracellular FA flux, a function particularly critical in oxidative tissues such as skeletal muscle with a high lipid turnover and metabolic demand[7]. Skeletal muscle is also a main site for postprandial glucose disposal, and muscle insulin resistance is a major risk factor of type 2 diabetes[8].

The LD surface is coated by perilipins and other structural proteins[1]. Enzymes involved in lipid metabolism such as lipases and lipogenic enzymes interact with LD. Perilipin 5 (PLIN5) belongs to the family of perilipins, and is highly expressed in oxidative tissues such as liver, heart, brown adipose tissue and skeletal muscle[9,10]. A recent study from Bosma and colleagues has described that overexpressing PLIN5 in mouse skeletal muscle increases intramyocellular TAG (IMTG) content[11], which is in agreement with other studies showing that PLIN5 acts as a lipolytic barrier to protect the LD against the hydrolytic activity of cellular lipases[12,13]. Interestingly,

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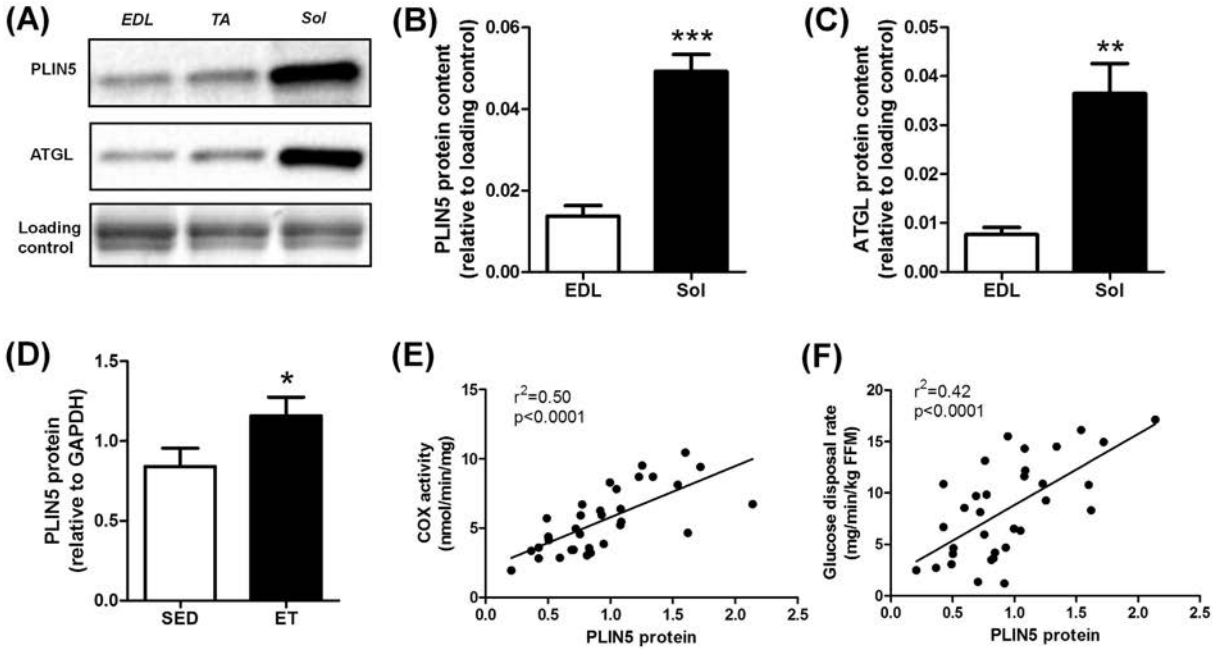

Figure 1. PLIN5 relates to oxidative capacity in mouse and human skeletal muscle. Representative blots (**A**) and quantification of PLIN5 (**B**) and ATGL (**C**) protein content in different mouse skeletal muscles (n = 5) (EDL: *extensor digitorum longus*, TA: *tibialis anterior*, Sol: *soleus*). $^{**}p < 0.01$, $^{***}p < 0.001$ versus EDL. (**D**) Quantification of PLIN5 protein content in *vastus lateralis* muscle of healthy lean and endurance-trained volunteers (n = 11 per group). Correlations between muscle PLIN5 protein and (**E**) cytochrome oxidase activity, and (**F**) glucose disposal rate (n = 33). $^{*}p < 0.05$ versus lean.

PLIN5 was also described to localize to mitochondria[14], and suggested to enhance FA utilization[15]. However, a protective role of PLIN5 against lipid-induced insulin resistance could not be confirmed after gene electroporation of PLIN5 in rat *tibialis anterior* muscle[11] and muscle-specific PLIN5 overexpression in mice[16]. In addition, a direct role of PLIN5 in facilitating FA oxidation upon increased metabolic demand has never been demonstrated in skeletal muscle.

To reconcile data from the literature, a hypothetical model would be that PLIN5 exhibits a dual role, buffering intracellular FA fluxes to prevent lipotoxicity in the resting state on one hand, and facilitating FA oxidation upon increased metabolic demand in the contracting state on the other hand. The aim of the current work was therefore to investigate the putative dual role of PLIN5 in the regulation of FA metabolism in skeletal muscle. The functional role of PLIN5 was studied *in vitro* in human primary muscle cells and *in vivo* in mouse skeletal muscle. Our data here reveal a key role of PLIN5 to adjust LD FA supply to metabolic demand, and also demonstrate that changes in PLIN5 expression influences lipotoxicity and insulin sensitivity in skeletal muscle.

Results

PLInN5 relates to oxidative capacity in mouse and human skeletal muscle. Muscle PLIN5 content was measured in various types of skeletal muscles in the mouse (Fig. 1A). We observed that PLIN5 was highly expressed in oxidative *soleus* muscle compared to mixed *tibialis anterior* or to the more glycolytic *extensor digitorum longus* muscle (3.6 fold, $p < 0.001$) (Fig. 1B). A similar expression pattern was observed for ATGL protein (4.7 fold, $p = 0.0019$) (Fig. 1C). In human *vastus lateralis* muscle, we observed a higher PLIN5 protein content in lean endurance-trained compared to lean sedentary individuals (+38%, $p = 0.033$) (Fig. 1D). A robust relationship between muscle PLIN5 and cytochrome oxidase activity, a marker of muscle oxidative capacity, was observed ($r^2 = 0.50$, $p < 0.0001$) (Fig. 1E). Significant positive correlations were also noted with citrate synthase activity ($r^2 = 0.42$, $p < 0.0001$) and β-hydroxy-acyl-CoA-dehydrogenase ($r^2 = 0.23$, $p = 0.0053$). Importantly, muscle PLIN5 protein show a strong positive association with glucose disposal rate measured during euglycemic hyperinsulinemic clamp in subjects with varying degrees of BMI and fitness ($r^2 = 0.42$, $p < 0.0001$) (Fig. 1F). Collectively, these data show that PLIN5 relates to muscle oxidative capacity and insulin sensitivity in mouse and human skeletal muscle.

PLIN5 overexpression reduces lipolysis and FA oxidation under basal connditions in human primary myotubes. Human skeletal muscle cells differentiated into myotubes are suited to perform mechanistic and metabolic studies[11]. However, PLIN5 mRNA expression is nearly undetectable in human primary myotubes compared to human muscle tissue (11 Ct difference, $2^{11} = 2054$ fold lower expression) (Supplemental Fig. S1). To recapitulate the ET phenotype *in vitro* in skeletal muscle cells, we overexpressed PLIN5 to gain further insight into its functional and metabolic role. Adenovirus-mediated PLIN5 overexpression led to a significant increase of PLIN5 protein content (3.6-fold, $p = 0.013$) (Fig. 2A). We first examined the effect of PLIN5

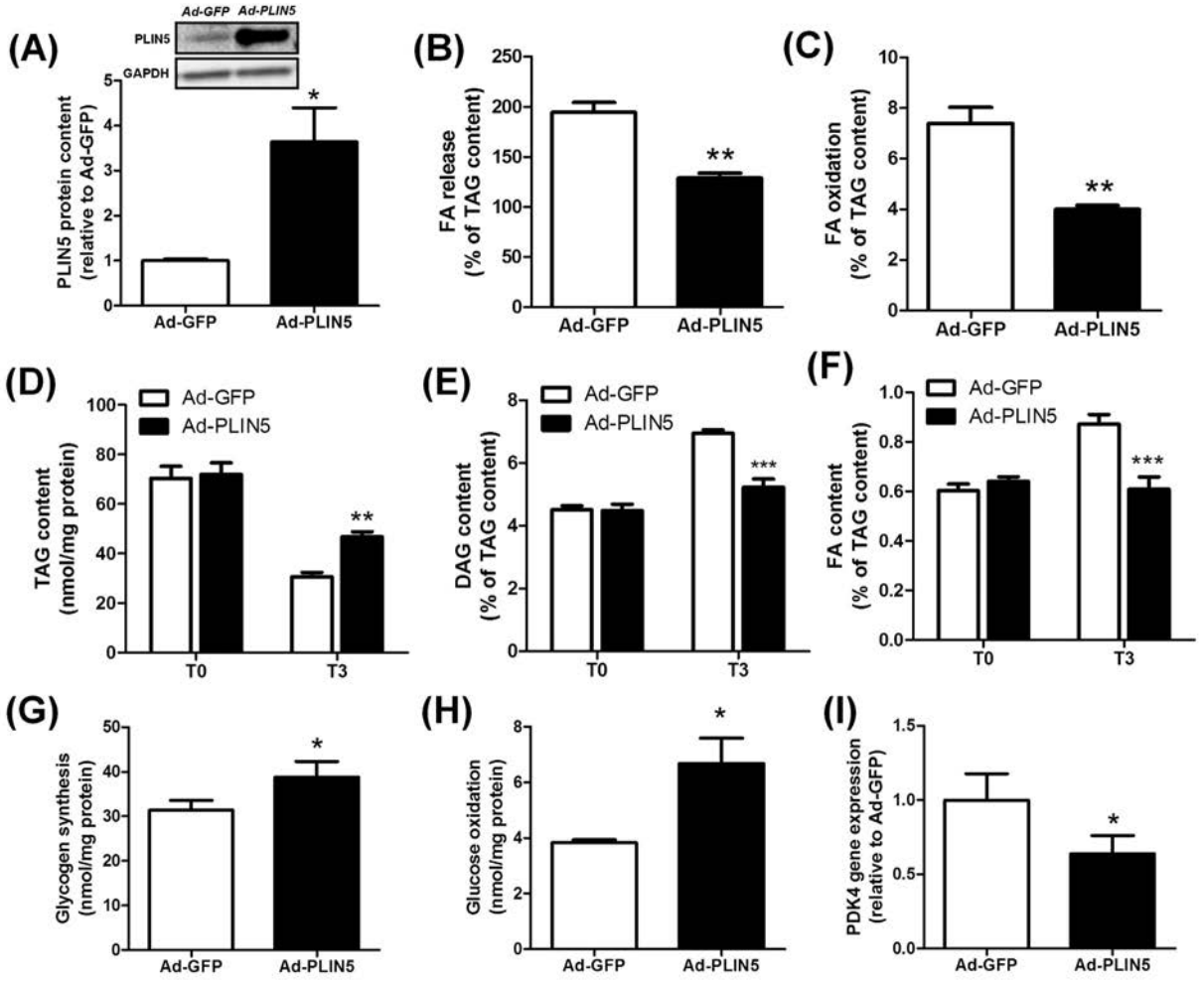

Figure 2. PLIN5 overexpression reduces lipolysis and FA oxidation under basal conditions in human primary myotubes. (**A**) Representative blot and quantification of PLIN5 protein content in control (Ad-GFP) and PLIN5-overexpressing myotubes (Ad-PLIN5) (n = 3). Pulse-Chase studies using [1-^{14}C] oleate were performed to determine (**B**) FA release into the culture medium (Ad-GFP = 59 ± 1.8 nmol/3 h/mg protein), (**C**) FA oxidation (Ad-GFP = 2.22 ± 0.13 nmol/3 h/mg protein), and the rate of incorporation of radiolabeled oleate into (**D**) TAG, (**E**) DAG (T0 Ad-GFP = 3.14 ± 0.14 nmol/3 h/mg protein) and (**F**) intracellular FA content (T0 Ad-GFP = 0.42 ± 0.03 nmol/3 h/mg protein) in control (Ad-GFP) and PLIN5-overexpressing myotubes (Ad-PLIN5). (**G**) Glycogen synthesis and (**H**) glucose oxidation were measured in control myotubes (Ad-GFP) and myotubes overexpressing PLIN5 (Ad-PLIN5) using [U-^{14}C] glucose. (**I**) PDK4 gene expression was measured in control (Ad-GFP) and PLIN5-overexpressing myotubes (Ad-PLIN5). (n = 6) *p < 0.05, **p < 0.01 ***p < 0.001 versus Ad-GFP.

overexpression on lipolysis and FA metabolism under basal condition, using a Pulse-Chase design. Endogenous TAG pool was pre-labeled (i.e. pulsed) overnight using [1-^{14}C] oleate. At the end of the pulse phase (i.e. T0), cells were chased for 3 h in a medium containing a low glucose concentration to promote lipolysis (i.e. T3). We found that PLIN5 overexpression decreased FA release into the culture medium (−34%, p = 0.044) (Fig. 2B), which was accompanied by a sharp reduction of FA oxidation compared to control cells (−46%, p = 0.0025) (Fig. 2C). We observed a 56% TAG depletion at T3 (i.e. after 3 hours of chase in a low-glucose medium) in control cells. PLIN5 overexpressing cells exhibited a lower TAG depletion rate compared to control cells (−38%, p = 0.0022) (Fig. 2D). Since the size of the TAG pool is a major determinant of TAG breakdown rate[17], lipid trafficking rates (FA and DAG) were normalized to TAG content. Consistently, intracellular DAG and FA accumulation during the chase period was totally abrogated by PLIN5 overexpression (Fig. 2E,F).

FA and glucose are the main nutrients competing for fuel oxidation in skeletal muscle[18]. By slowing down lipid utilization, PLIN5 overexpression enhanced basal glycogen synthesis (+24%, p = 0.045) (Fig. 2G) and glucose oxidation (+74%, p = 0.010) (Fig. 2H). As previously observed in this cell model system[19], this metabolic switch was paralleled by a significant down-regulation of *pyruvate dehydrogenase kinase 4* (PDK4) (−36%, p = 0.024) (Fig. 2I). Taken together, these results clearly show that PLIN5 overexpression slows down lipolysis and FA oxidation and favors a switch towards glucose metabolism in human muscle cells.

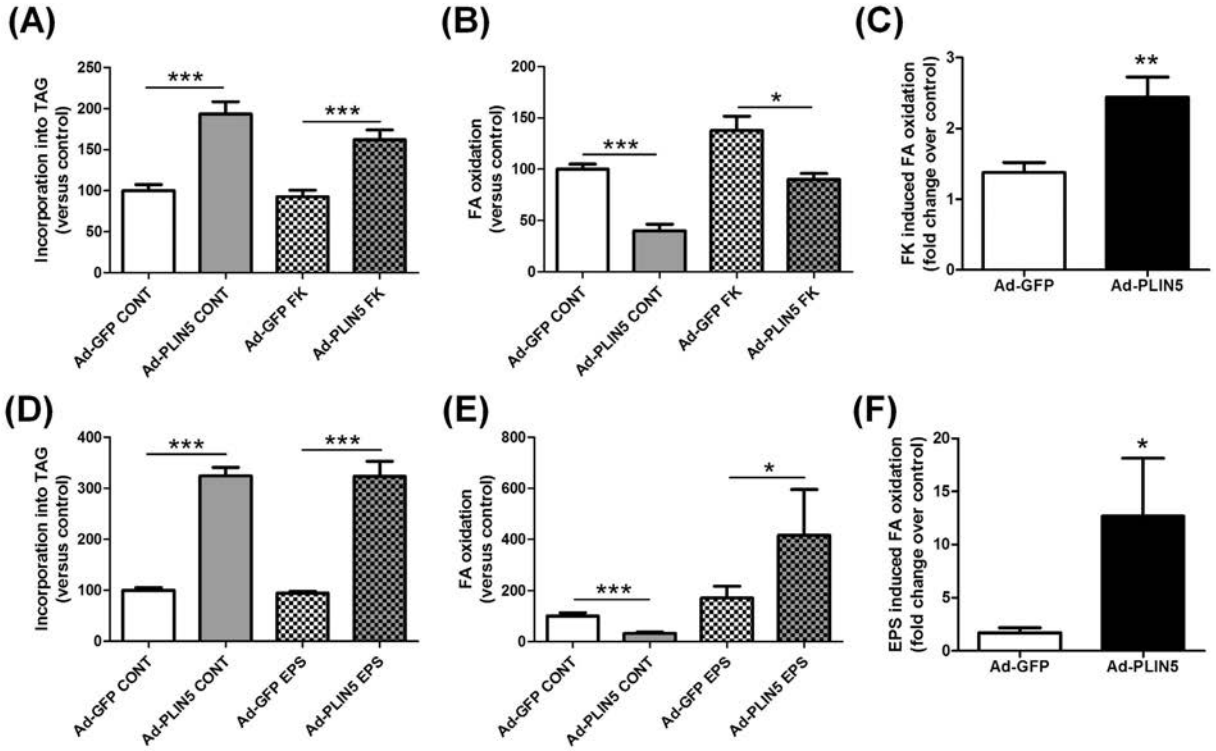

Figure 3. PLIN5 overexpression facilitates lipid oxidation upon increased metabolic demand. Pulse-Chase studies using [1-^{14}C] oleate were performed to determine the rate of (**A,D**) incorporation of radiolabeled oleate into TAG and (**B,E**) oleate oxidation in control myotubes (Ad-GFP) and myotubes overexpressing PLIN5 (Ad-PLIN5) either during (**A–C**) forskolin (FK) (Ad-GFP CONT = 1.66 ± 0.25 nmol/3 h/mg protein) or (**D–E**) electrical pulse (EPS) stimulation (Ad-GFP CONT = 6.33 ± 2.05 nmol/24 h/mg protein) (n = 6). Values are expressed in % of Ad-GFP Control (**A,B,D,E**) and in fold change over control in (**C** and **F**). *$p < 0.05$, **$p < 0.01$ ***$p < 0.001$ versus Ad-GFP.

PLIN5 overexpression facilitates lipid oxidation upon increased metabolic demand. Considering that PLIN5 is elevated in skeletal muscle of athletes with a high lipid turnover, we investigated its role under stimulation of lipolysis, increased TAG turnover and metabolic demand in human primary myotubes. Thus PLIN5-mediated TAG accumulation (+93%, $p = 0.0002$) was reduced in the presence of forskolin (a potent lipolysis activator) (+75%, $p = 0.0006$) (Fig. 3A). In line with this, FA oxidation was reduced by 60% in PLIN5 overexpressing myotubes under basal conditions ($p < 0.0001$), while this decrease was of only 35% upon forskolin stimulation ($p = 0.01$) (Fig. 3B). Overall, PLIN5 overexpression greatly potentiated forskolin-induced FA oxidation when compared to control cells (+77%, $p = 0.007$) (Fig. 3C).

Because muscle contraction represents a more physiological stimulation of FA metabolism and increased metabolic demand, we used a model of electrical pulse stimulation (EPS) to recapitulate contraction-mediated lipolysis *in vitro*. As a model validation, we observed no significant change in total glycogen content and a sharp increase of FA oxidation, which represent classical skeletal muscle physiological adaptations to endurance training (REF). These effects were accompanied by a robust induction of interleukin-6 gene expression, a well-known exercise-induced myokine (Supplemental Fig. S2). Interestingly, despite no major change in TAG pools under basal or stimulated conditions (Fig. 3D), we observed a very sharp increase of EPS-mediated FA oxidation in PLIN5 overexpressing cells (2.4 fold, $p = 0.014$) (Fig. 3E). Importantly, we observed that EPS increased FA oxidation by 1.7 fold in control myotubes, while this effect was robustly enhanced up to 12.7 fold in PLIN5 overexpressing cells (Fig. 3F). Together, this suggests for the very first time that PLIN5 is necessary to boost TAG lipolysis and FA oxidation upon increased metabolic demand in skeletal muscle.

PLIN5 exert a protective role against palmitate-induced lipotoxicity. Besides a key role in controlling LD lipolysis, PLIN5 may sequester toxic lipids into LD and reduce intracellular lipotoxic insults[20]. To test this hypothesis, we challenged myotubes with palmitate at a concentration known to induce lipotoxicity and insulin resistance[21]. As a model validation, we first observed that palmitate treatment strongly elevated total diacylglycerols (+4.7 fold, $p < 0.0001$) and ceramides (+4 fold, $p < 0.0001$) levels while inhibiting insulin-mediated glycogen synthesis (−37%, $p = 0.016$) (Fig. 4). Of note, PLIN5 overexpressing myotubes were partly protected from palmitate-mediated insulin resistance and lipotoxicity. Insulin-stimulated glycogen synthesis was higher in PLIN5 overexpressing myotubes challenged with palmitate compared to control myotubes (+26%, $p = 0.0025$) (Fig. 4A). Similarly, palmitate-mediated DAG accumulation was slightly reduced in PLIN5 overexpressing myotubes (−16%, $p = 0.039$) (Fig. 4B). Finally, PLIN5 overexpressing myotubes displayed reduced concentrations

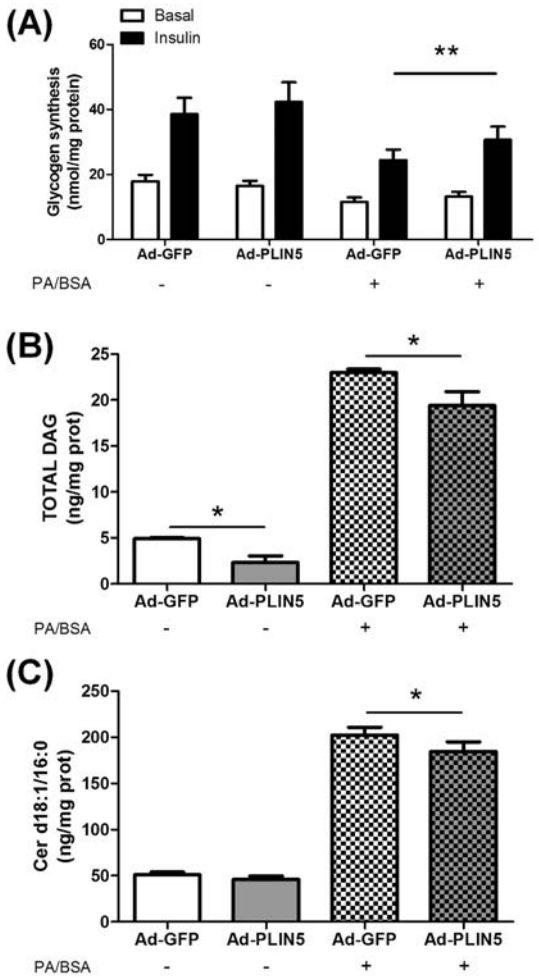

Figure 4. PLIN5 exerts a protective role against palmitate-induced lipotoxicity. (**A**) Glycogen synthesis was measured in control myotubes (Ad-GFP) and myotubes overexpressing PLIN5 (Ad-PLIN5) using [U-^{14}C] glucose in absence or presence of 100 nM insulin, in control cells and in cells treated with 300 μM of palmitic acid for 24 h (n = 9). (**B**) Total diacylglycerols (DAG) and (**C**) Ceramide (CER) d18:1/16:0 content were measured in control myotubes (Ad-GFP) and myotubes overexpressing PLIN5 (Ad-PLIN5) (n = 4). *$p < 0.05$, **$p < 0.01$ versus Ad-GFP.

of all ceramides species measured in response to palmitate treatment (Two-way ANOVA $p = 0.04$), particularly due to reduced ceramide d18:1/16:0 content (Fig. 4C), the most abundant ceramide species in our cell model. Collectively, these results highlight a slight protective role of PLIN5 against lipotoxicity and palmitate-induced insulin resistance in muscle cells.

PLIN5 knockdown in mouse skeletal muscle increases lipid oxidation and reduces insulin-stimulated glucose uptake under normal chow diet. Considering that PLIN5 is strongly expressed in skeletal muscle and that previous gain-of-function studies in muscle failed to substantiate the causal and mechanistic link between PLIN5 and insulin sensitivity, we assessed the physiological role of PLIN5 *in vivo* by inducing a muscle-restricted loss-of-function. We knocked down its expression by injecting an AAV1 containing a shRNA directed against PLIN5 in *tibialis anterior* muscle of 10-week old C57BL/6 J mice. Intramuscular AAV1-shRNA-PLIN5 injection significantly reduced PLIN5 mRNA expression (−23%, $p = 0.014$) (Fig. 5A) and protein content (−21%, $p = 0.024$) (Fig. 5B) compared to the contralateral leg injected with an AAV1 containing a non-targeted shRNA. Of note, no functional compensation by other PLIN isoforms was observed in PLIN5 knocked down muscles (Supplemental Fig. S3). In agreement with *in vitro* data in the basal state, knockdown of PLIN5 increased the rate of FA oxidation to CO_2 (+51%) and ASM (i.e. acid soluble metabolites) (+21%) ($p < 0.05$) (Fig. 5C). No change in glucose oxidation was observed (Fig. 5D). Since PLIN5 null mice exhibit signs of insulin resistance in skeletal muscle[22], we next measured insulin-stimulated glucose uptake. Interestingly, PLIN5 knockdown decreased insulin-stimulated muscle glucose uptake (−27%, $p = 0.0003$) (Fig. 5E). However, muscle insulin resistance appeared independent of significant change in total (shNT 0.11 ± 0.01 *vs.* shPLIN5 0.12 ± 0.02 nmol/mg, NS) and various ceramide species. Taken together, our data argue for a physiological role of PLIN5 in the regulation of FA oxidation and insulin sensitivity in skeletal muscle *in vivo*.

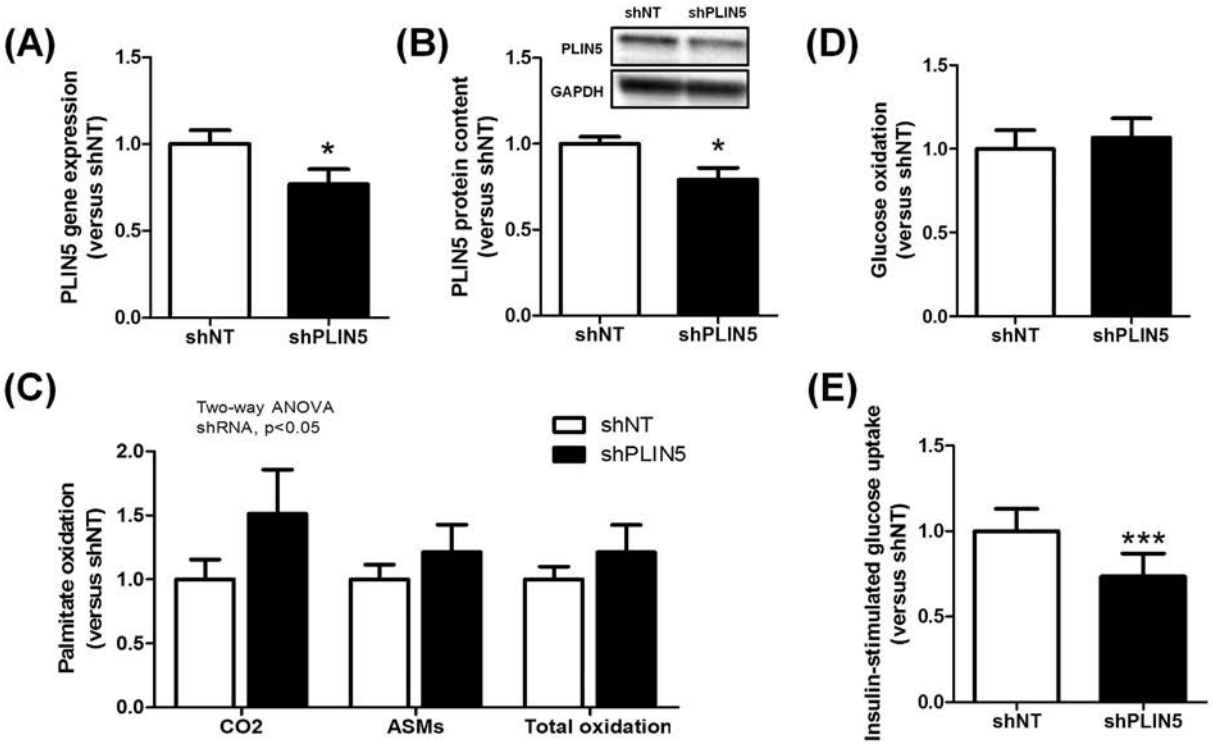

Figure 5. PLIN5 knockdown in mouse skeletal muscle increases lipid oxidation and reduces insulin-stimulated glucose uptake under normal chow diet. PLIN5 (**A**) gene expression and (**B**) protein content measured in control (shNT) and PLIN5 silenced (shPLIN5) mouse *tibialis anterior* muscle (n = 6). Palmitate (**C**) and glucose (**D**) oxidation rate were measured using respectively [U-^{14}C] glucose or [1-^{14}C] palmitate in control (shNT) and PLIN5 silenced (shPLIN5) muscle homogenates. Palmitate oxidation (i.e. CO2), acid soluble metabolites accumulation (i.e. ASMs) and total oxidation (i.e. the sum of CO2 release and ASMs accumulation) were measured (n = 6). (**E**) Insulin-stimulated glucose uptake was determined in control (shNT) and PLIN5 knockdown (shPLIN5) muscles. (n = 7). *$p < 0.05$, ***$p < 0.001$ versus shNT.

PLIN5 knockdown in mouse skeletal muscle ameliorates insulin action under high-fat feeding. We next investigated the impact of PLIN5 knockdown in *tibialis anterior* muscle under high fat diet feeding for 12 weeks. Intramuscular AAV1-shRNA-PLIN5 injection in HFD-fed mice reduced PLIN5 mRNA level by 31% ($p = 0.015$) (Fig. 6A) and protein content by 54% ($p = 0.0003$) (Fig. 6B), without any compensatory changes in the expression level of PLIN2, PLIN3 and PLIN4 (Supplemental Fig. S3). In contrast with normal chow diet-fed mice, insulin-stimulated muscle glucose uptake was improved in PLIN5 knocked down legs of HFD-fed mice (+37%, $p = 0.0031$) (Fig. 6C). This was accompanied by a decrease in total ceramide content (−18%, $p = 0.028$) (Fig. 6D), while DAG content remained unchanged (Fig. 6E). In agreement, we also observed a significant increase of insulin-stimulated Akt phosphorylation on serine 473 and threonine 308 in PLIN5 knockdown muscle compared to the contralateral leg (1.7 fold and 2.1 fold, respectively, $p = 0.048$) (Fig. 6F). Collectively, while PLIN5 knock-down promotes insulin resistance in skeletal muscle of chow-fed mice, it paradoxically partly protects skeletal muscle against HFD-induced insulin resistance.

High-fat feeding up-regulates PLIN5 in skeletal muscle independently of PPARβ. PLIN5 has been described as a *Peroxisome Proliferator-Activated Receptors* (PPAR)-target gene in a mouse muscle cell line model[23]. Since we noted a striking up-regulation of PLIN5 with high-fat feeding at both mRNA and protein levels (Supplemental Fig. S4), we examined PLIN5 regulation by PPAR *in vitro* and *in vivo*. We confirmed previous findings[23] showing that PLIN5 is a PPARβ-responsive gene in human primary myotubes (Supplemental Fig. S4). Interestingly, PLIN5 was specifically induced by a PPARβ agonist (GW0742) in this cell model system (5.3 fold, $p < 0.001$). We next investigated whether HFD-mediated up-regulation of PLIN5 was mediated by activation of PPARβ in skeletal muscle *in vivo*. Of interest, muscle PLIN5 protein content was similar in PPARβ knockout mice, while HFD-mediated up-regulation of PLIN5 was unaffected in PPARβ knockout mice (Supplemental Fig. S4). Thus, HFD-mediated up-regulation of PLIN5 could be seen as an adaptive response to facilitate fat storage into LD of excess incoming FA and minimize lipotoxicity. Although PLIN5 is a PPARβ-responsive gene in skeletal muscle, HFD-mediated up-regulation of PLIN5 appears independent of PPARβ.

Discussion

LD play a critical role in oxidative tissues to maintain appropriate fuel supply during periods of energy needs but also to buffer daily fluxes of FA to avoid cellular lipotoxicity. PLIN5 has been previously shown as a LD protein

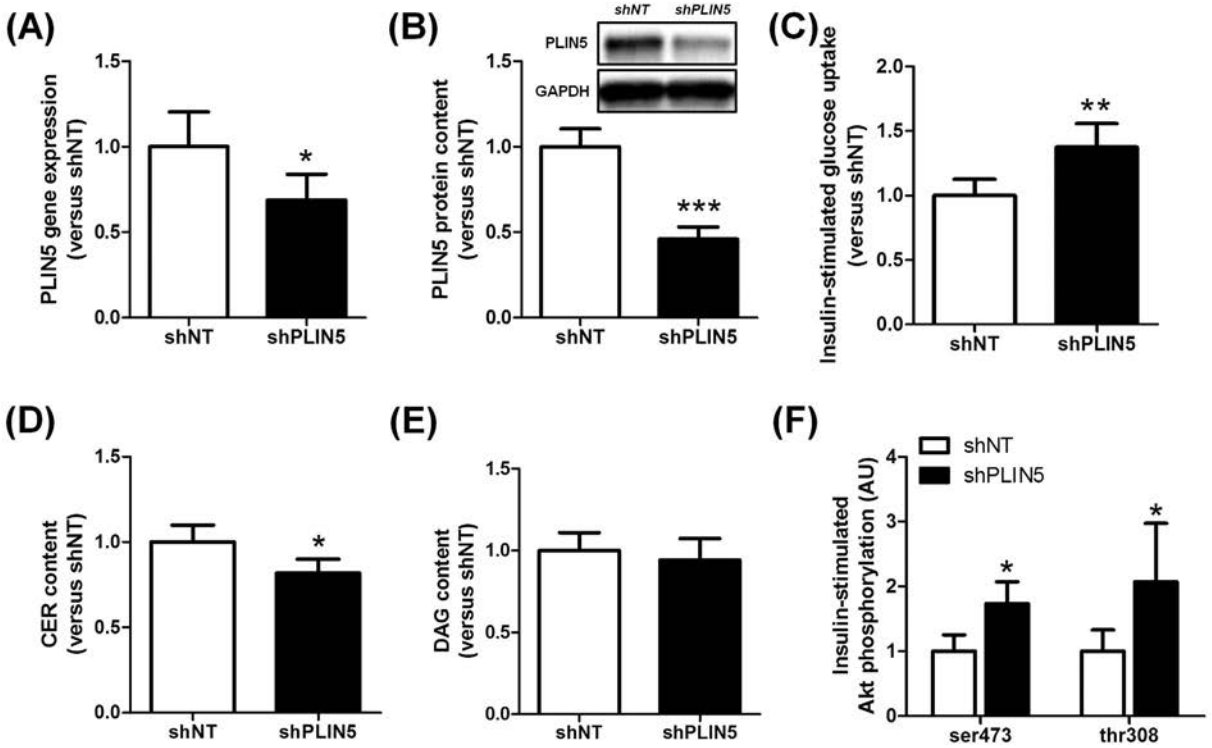

Figure 6. PLIN5 knockdown in mouse skeletal muscle ameliorates insulin action under high-fat feeding. PLIN5 (**A**) gene expression and (**B**) protein content measured in control (shNT) and PLIN5 silenced (shPLIN5) mouse *tibialis anterior* muscle (n = 6). (**C**) Insulin-stimulated glucose uptake, (**D**) total ceramide (CER) and (**E**) total diacylglycerols (DAG) content were determined in control (shNT) and PLIN5 knockdown (shPLIN5) muscles. (n = 7). (**F**) Insulin-stimulated Akt phosphorylation on Ser473 and Thr308 residues was measured in control (shNT) and PLIN5 silenced (shPLIN5) muscle (n = 4). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus shNT.

inhibiting lipolysis and correlating with insulin sensitivity[13,24,25]. The current work demonstrates for the first time that PLIN5 protects against palmitate-induced insulin resistance and facilitates FA oxidation in response to muscle contraction and increased metabolic demand *in vitro*. We further show a causal link between down-regulation of PLIN5 and insulin resistance *in vivo* in mouse skeletal muscle. We show here that the skeletal muscle enriched PLIN5 protein has a key role in controlling fat oxidation and lipotoxicity by fine tuning FA fluxes in and out of the LD from the resting to the contracting state. PLIN5 facilitates fat storage into LD and inhibits FA oxidation in the resting state while sharply boosting IMTG lipolysis and FA oxidation during muscle contraction or PKA stimulation (Fig. 7). Although the precise molecular mechanism was not investigated here, one can speculate that PLIN5 is physically relocated out of the LD to favor LD hydrolysis by adipose triglyceride lipase and FA channeling into mitochondria[15].

We first observed that PLIN5 tightly correlates with oxidative capacity of mouse and human skeletal muscle. Muscle PLIN5 content strongly correlated as well with whole-body insulin sensitivity. In addition, we confirm that endurance-trained subjects exhibited higher levels of PLIN5 protein compared to lean sedentary subjects as previously described[24]. This is in line with various studies showing that aerobic exercise training increases PLIN5 protein, oxidative capacity and insulin sensitivity in skeletal muscle[26–28]. Thus endurance-trained individuals display higher lipid content, oxidative capacity and insulin sensitivity compared to matched sedentary controls[29,30]. We next observed that PLIN5 overexpression in human primary myotubes has a modest protective effect against saturated fat-induced lipotoxicity and insulin-resistance. Thus PLIN5 seems to preserve insulin action (glycogen synthesis) by sequestering toxic saturated lipids into LDs[31,32]. Our data are in agreement with a recent study showing that PLIN5 overexpression in C2C12 mouse myotubes facilitate palmitate sequestration into LD and remodels their lipid composition[20]. Finally, a recent study by Mason and colleagues reported that PLIN5 knockout mice develop insulin resistance associated with ceramide accumulation in skeletal muscle[22].

Studies from different groups have shown that PLIN5 overexpression increases TAG storage in mouse skeletal[11] and cardiac[12] muscle. We observed here that PLIN5 overexpression slows down TAG-derived lipolysis and FA oxidation in basal resting conditions, and concomitantly induces a switch towards glucose utilization. Of importance, two reports described that PLIN5 not only localizes to the LD surface, but also to the mitochondria[14,15]. We show here for the first time that PLIN5 overexpression in human primary myotubes sharply enhanced FA oxidation upon forskolin- and contraction-induced lipolysis activation and metabolic demand. This suggests that PLIN5 might provide a physical linkage between LD and mitochondria in a context of increased energy demand. Our data are in agreement with data in ALM12 liver cells in which PLIN5 overexpression enhanced FA release when lipolysis was activated by the adenylyl cyclase activator forskolin[15]. PLIN5 appears to be phosphorylated by PKA (cAMP-dependent protein kinase)[33], in a similar fashion as PLIN1 in adipose tissue[34]. The molecular

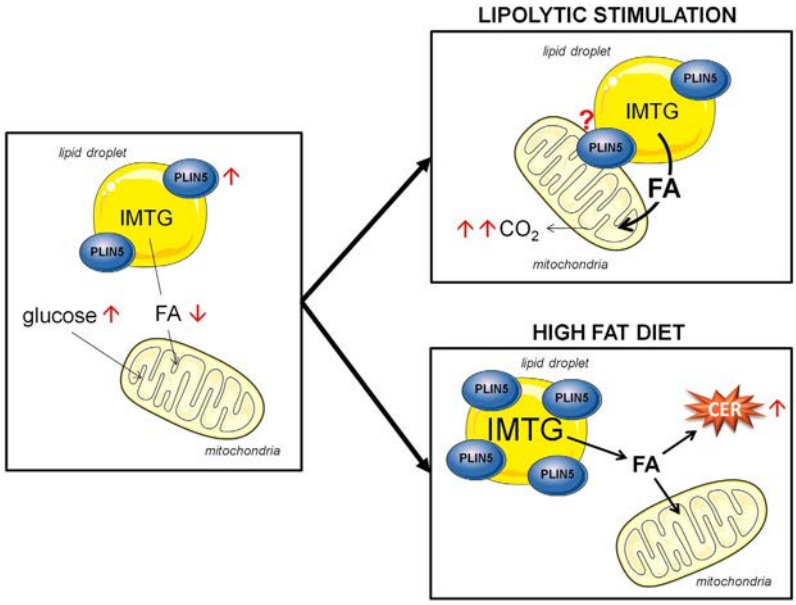

Figure 7. Proposed mechanistic model of PLIN5 in skeletal muscle upon various metabolic states. In the resting state, PLIN5 protects LD from lipolytic attack by lipases. An increase in PLIN5 content (red arrows) slows down lipolysis and FA oxidation, favoring a switch towards glucose utilization. During lipolytic stimulation (i.e. PKA activation or contraction), PLIN5 enhances FA oxidation, thereby increasing CO_2 production. It has been suggested that PLIN5 could provide a physical linkage between LD and mitochondria. We can hypothesize that this relocation has metabolic consequences by facilitating FA channeling from LD to mitochondria, thus allowing a more efficient coupling between IMTG lipolysis and FA oxidation upon increased metabolic demand. Finally, the up-regulation of PLIN5 with high-fat feeding is insufficient to protect from LD-mediated CER accumulation. FA: Fatty Acids; IMTG: Intramyocellular Triacylglycerols; CER: Ceramides.

pathways induced by contraction converging to PLIN5 where not investigated here and require a detailed examination in future studies.

We next investigated the physiological role of PLIN5 in skeletal muscle *in vivo* through loss-of-function studies using AAV gene delivery. PLIN5 was knocked down in one leg of mice fed either standard chow or high fat diets. In line with our *in vitro* data, PLIN5 knockdown induced a compensatory increase of FA oxidation rate under standard chow diet. This could be explained by a better access of lipases to LD and greater TAG turnover as shown in cardiac muscle of PLIN5-deficient mice[35]. Interestingly, it has been described that a global PLIN5 deficiency induces muscle insulin resistance[22]. However, this effect may be confounded by systemic factors. In line with the positive association between muscle PLIN5 content and insulin sensitivity observed in humans, we show that a partial down-regulation of PLIN5 in mouse skeletal muscle inhibits insulin-stimulated glucose uptake. This highlights for the first time that down-regulation of PLIN5 promotes insulin resistance in a muscle-autonomous fashion. This might be explained by a deficient coupling between FA supply and mitochondrial oxidation resulting in muscle inflammation, although further work is needed to better understand these mechanisms.

Under HFD, we first observed a striking up-regulation of PLIN5 mRNA and protein levels in skeletal muscle. We describe here PLIN5 as a PPARβ target gene in human primary skeletal muscle cells, which is in agreement with data from C2C12 mouse myotubes[23]. However, we show for the first time that baseline expression of muscle PLIN5 is not influenced by PPARβ, and that HFD-mediated up-regulation of PLIN5 is not driven by PPARβ. Although baseline expression of muscle PLIN5 is strongly reduced in PPARα knockout mice, HFD-mediated up-regulation of PLIN5 is not prevented as well in these transgenic mice[23]. It is still unclear how HFD promotes the up-regulation of muscle PLIN5 but this may be under the control of PPARγ which contribute to lipid accumulation in skeletal muscle during high fat feeding[36]. Other transcription factors related to lipid storage may be involved. Thus, HFD-mediated up-regulation of PLIN5 seems to be an adaptive mechanism to favor FA sequestering and accumulation into TAG pools. However, and contrary to our expectations, muscle PLIN5 knockdown in a context of high fat diet improved muscle insulin sensitivity. It has been previously shown that high fat feeding is accompanied by an upregulation of muscle oxidative capacity which does not appear sufficient to prevent both TAG accumulation and lipotoxicity[37,38]. Thus, lower levels of PLIN5 during HFD, achieved by AAV-mediated knockdown, might in this context facilitate IMTG lipolysis and FA utilization in a resting muscle with high lipid content, and therefore reduce lipotoxicity. This hypothesis is partly supported by the observation of a reduced total ceramide content and an increase of Akt Ser473 and Thr308 phosphorylation, while no change in total DAG was observed in PLIN5-knocked down muscle of HFD-fed mice. These data are consistent with at least another study showing that PLIN5 knockout mice display a markedly improved glucose tolerance under HFD with a trend toward increased peripheral glucose clearance[22]. Thus our data brings light on this previous observation showing an elevated rate of glucose uptake in PLIN5-deficient skeletal muscles. Overall, although PLIN5 exhibits

a protective role against lipotoxicity in standard nutritional conditions, HFD-mediated up-regulation of PLIN5 appears deleterious for the maintenance of insulin action in skeletal muscle.

In summary, we provide mechanistic evidences that PLIN5 plays a key role in skeletal muscle. We show for the first time a dual role of PLIN5, favoring TAG accumulation and protecting from high intracellular toxic lipid levels in the resting state, while facilitating IMTG lipolysis and FA oxidation during contraction and increased metabolic demand. This work further highlights the important role of LD function and dynamics for metabolic regulation and for the maintenance of insulin sensitivity in skeletal muscle.

Methods

Human muscle sampling. Data and samples from men aged between 34 and 53 years with varying degree of BMI and insulin sensitivity were available from a prior study (n = 33)[39]. Of these, 11 were normal weight sedentary controls, 11 were obese sedentary and 11 were normal weight endurance-trained individuals. The overall study design and subject testing have been partly described in[39]. The study was performed according to the latest version of the Declaration of Helsinki and the Current International Conference on Harmonization (ICH) guidelines. The research protocol was approved by the Université Laval ethics committee and all subjects provided written informed consent. Samples of *vastus lateralis* (~40 mg) were obtained, blotted free of blood, cleaned to remove fat and connective tissue and snap-frozen in liquid nitrogen for Western blot analyses. All samples were stored at −80 °C under argon or nitrogen gas until use.

Skeletal muscle primary cell culture. Satellite cells from *rectus abdominis* of healthy male subjects (age 34.3 ± 2.5 years, BMI 26.0 ± 1.4 kg/m^2, fasting glucose 5.0 ± 0.2 mM) were kindly provided by Prof. Arild C. Rustan (Oslo University, Norway). Satellite cells were isolated by trypsin digestion, preplated on an uncoated petri dish for 1 h to remove fibroblasts, and subsequently transferred to T-25 collagen-coated flasks in Dulbecco's Modified Eagle's Medium (DMEM) low glucose (1 g/L) supplemented with 10% FBS and various factors (human epidermal growth factor, BSA, dexamethasone, gentamycin, fungizone, fetuin) as previously described[40]. Cells from several donors were pooled and grown at 37 °C in a humidified atmosphere of 5% CO_2. Differentiation of myoblasts (i.e. activated satellite cells) into myotubes was initiated at ~80–90% confluence, by switching to α-Minimum Essential Medium with 2% penicillin-streptomycin, 2% FBS, and fetuin (0.5 mg/ml). The medium was changed every other day and cells were grown up to 5 days. For pharmacological treatments, cells were exposed to a PPARα or PPARβ agonist (GW7647 and GW0742, respectively) or a PPARβ antagonist (GSK0660) for 24 h at the end of the differentiation.

Overexpression of PLIN5 in human myotubes. For overexpression experiments, adenoviruses expressing in tandem GFP and human PLIN5 (hPLIN5) were used (Vector Biolabs, Philadelphia, PA). Control was performed using adenoviruses containing GFP gene only. Myotubes were infected with both adenoviruses at day 4 of differentiation and remained exposed to the virus for 24 h in serum-free DMEM containing 100 μM of oleate complexed to BSA (ratio 2/1). Oleate was preferred to palmitate for lipid loading of the cells, to favor triacylglycerol (TAG) synthesis and to avoid the intrinsic lipotoxic effect of palmitate[41]. As a model of lipid-induced lipotoxicity and insulin resistance, oleate was replaced by palmitate in some experiments to metabolically challenge the cells.

Animal studies. All experimental procedures were approved by a local ethics committee (CEEA122 INSERM US006/CREFRE, protocol n°C14/U1048/DL/13) and performed according to INSERM animal care facility guidelines and to the 2010/63/UE European Directive for the care and use of laboratory animals. Sixteen week-old male PPARβ knockout and wild-type mice on a SV129/C57Bl6 background were used for muscle tissue collection.

Four-week-old C57BL/6 J male mice were housed in a pathogen-free barrier facility (12 h light/dark cycle) and fed either normal chow diet (10% calories from fat) (D12450J, Research Diets, New Jersey) or high-fat diet (60% calories from fat) (D12492, Research Diets, New Jersey). To induce an *in vivo* knockdown of PLIN5 specifically in skeletal muscle, mice were injected with 1×10^{11} GC (i.e. genome copy) of AAV1 vector (Vector Biolabs, Philadelphia, PA) in *tibialis anterior* muscles at 10 weeks of age. Each mouse had one leg injected with AAV1-shPLIN5 and the contralateral leg injected with AAV1-shNT (nontarget) as a control. Six weeks following the injections, mice were killed by cervical dislocation and muscles (i.e. *tibialis anterior* and *extensor digitorum longus*) were dissected and either used *ex-vivo* for palmitate and glucose oxidation assays or stored at −80 °C for protein, RNA and lipid analyses.

Real-time RT-qPCR. Total RNA from cultured myotubes or *tibialis anterior* muscle was isolated using Qiagen RNeasy mini kit according to manufacturer's instructions (Qiagen GmbH, Hilden, Germany). The quantity of RNA was determined on a Nanodrop ND-1000 (Thermo Scientific, Rockford, IL, USA). Reverse-transcriptase PCR was performed on a Techne PCR System TC-412 using the Multiscribe Reverse Transcriptase method (Applied Biosystems, Foster City, CA). Real-time quantitative PCR (qPCR) was performed to determine cDNA content. All primers were bought from Applied Biosystems and were: 18 S (Taqman assay ID: Hs99999901_s1), PLIN5 (Hs00965990_m1 and Mm00508852_m1), and PDK4 (Hs01037712_m1). The amplification reaction was performed in duplicate on 10ng of cDNA in 96-well reaction plates on a StepOnePlus™ system (Applied Biosystems). All expression data were normalized by the $2^{(\Delta Ct)}$ method using 18 S as internal control.

Western blot analysis. Muscle tissues and cell extracts were homogenized in a buffer containing 50 mM HEPES, pH 7.4, 2 mM EDTA, 150 mM NaCl, 30 mM $NaPPO_4$, 10 mM NaF, 1% Triton X-100, 1.5 mg/ml benzamidine HCl and 10 μl/ml of each: protease inhibitor, phosphatase I inhibitor and phosphatase II inhibitor (Sigma-Aldrich). Tissue homogenates were centrifuged for 25 min at 15,000 *g* and supernatants were stored at

−80 °C. A total of 30 μg of solubilized proteins from muscle tissue and myotubes were run on a 4–12% SDS-PAGE (Biorad), transferred onto nitrocellulose membrane (Hybond ECL, Amersham Biosciences), and blotted with the following primary antibodies: PLIN5 (#GP31, Progen), ATGL (#2138, Cell Signaling Technology Inc.), Akt (#4691, Cell Signaling Technology Inc.), pAkt S473 (#4060, Cell Signaling Technology Inc.), pAkt T308 (#2965, Cell Signaling Technology Inc.). Subsequently, immunoreactive proteins were blotted with secondary HRP-coupled antibodies (Cell Signaling Technology Inc.) and revealed by enhanced chemiluminescence reagent (SuperSignal West Femto, Thermo Scientific), visualized using the ChemiDoc MP Imaging System and data analyzed using the ImageLab 4.2 version software (Bio-Rad Laboratories, Hercules, USA). GAPDH (#2118, Cell Signaling Technology Inc.) was used as an internal control.

Determination of glucose metabolism. Cells were pre-incubated with a glucose- and serum-free medium for 90 min, then exposed to DMEM supplemented with D[U-^{14}C] glucose (1 μCi/ml; PerkinElmer, Boston, MA). Following incubation, glucose oxidation was determined by counting of $^{14}CO_2$ released into the culture medium. The cells were then solubilized in KOH 30% and glycogen synthesis was determined as previously described[42]. Total glycogen content was determined spectrophotometrically after complete hydrolysis into glucose by the α-amiloglucosidase as previously described[43].

Determination of fatty acid metabolism. Cells were pulsed overnight for 18 h with [1-^{14}C] oleate (1 μCi/ml; PerkinElmer, Boston, MA) and cold oleate (100 μM) to prelabel the endogenous TAG pool. Oleate was coupled to FA-free BSA in a molar ratio of 5:1. Following the pulse, myotubes were chased for 3 h in DMEM containing 0.1 mM glucose, 0.5% FA-free BSA, and 10 μM triacsin C to block FA recycling into the TAG pool as described elsewhere[44], in absence or presence of 10 μM forskolin to stimulate lipolysis. For electrical pulse stimulation experiments, cells were chased for 24 h in DMEM containing 1 mM glucose, 0.5% FA-free BSA and 10 μM triacsin C while electrically stimulated by 2 ms pulses at a frequency of 0.1 Hz. TAG-derived FA oxidation was measured by the sum of $^{14}CO_2$ and ^{14}C-ASM (acid soluble metabolites) in absence of triacsin C as previously described[40]. Myotubes were harvested in 0.2 ml SDS 0.1% at the end of the pulse and of the chase period to determine oleate incorporation into TAG and protein content. The lipid extract was separated by TLC using heptane-isopropylether-acetic acid (60:40:4, v/v/v) as developing solvent. All assays were performed in duplicates, and data were normalized to cell protein content. Palmitate oxidation rate was measured as previously described[43].

Tissue-specific [2-^{3}H] deoxyglucose uptake *in vivo*. Muscle-specific glucose uptake was assessed in response to an intraperitoneal bolus injection of 2-[1,2-^{3}H(N)]deoxy-D-Glucose (PerkinElmer, Boston, Massachusetts) (0.4μCi/g body weight) and insulin (3 mU/g body weight). The dose of insulin was determined in preliminary studies to reach a nearly maximal stimulation of insulin signaling and glucose uptake in all muscle types and metabolic tissues. Mice were killed 30 min after injection and tissues were extracted by precipitation of 2-deoxyglucose-6-phosphate as previously described[45].

Determination of neutral lipid and ceramide content. Triacylglycerols and diacylglycerols were determined by gas chromatography, and ceramide and sphingomyelin species by high-performance liquid chromatography-tandem mass spectrometry after total lipid extraction as described elsewhere[45,46].

Statistical analyses. All statistical analyses were performed using GraphPad Prism 5.0 for Windows (GraphPad Software Inc., San Diego, CA). Normal distribution and homogeneity of variance of the data were tested using Shapiro-Wilk and F tests, respectively. One-way ANOVA followed by Tukey's post hoc tests and Student's *t*-tests were performed to determine differences between treatments. Two-way ANOVA and Bonferroni's post hoc tests were used when appropriate. All values in figures and tables are presented as mean ± SEM. Statistical significance was set at $p < 0.05$.

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Author Contributions

C.L., V.B., A.M., K.L., P.M.B., E.M., A.M., A.M., A.T., S.J.W., H.G., D.L., D.R.J. and C.M. researched data and edited the manuscript. C.L. and C.M. wrote the manuscript. Dr. Cedric Moro is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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SUPPPLEMENTARY INFORMATION

Perilipin 5 fine-tunes lipid oxidation to metabolic demand and protects against lipotoxicity in skeletal muscle

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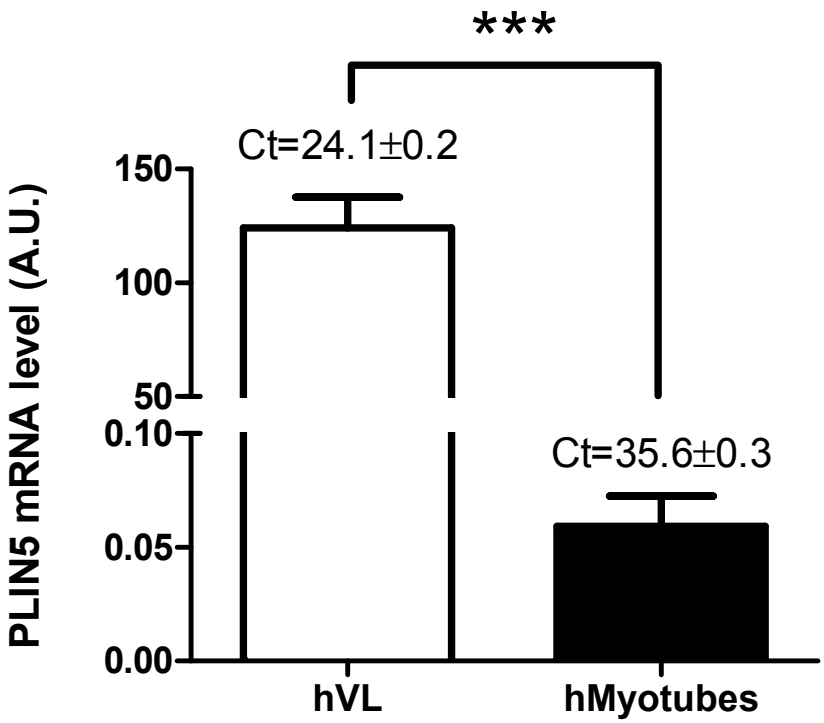
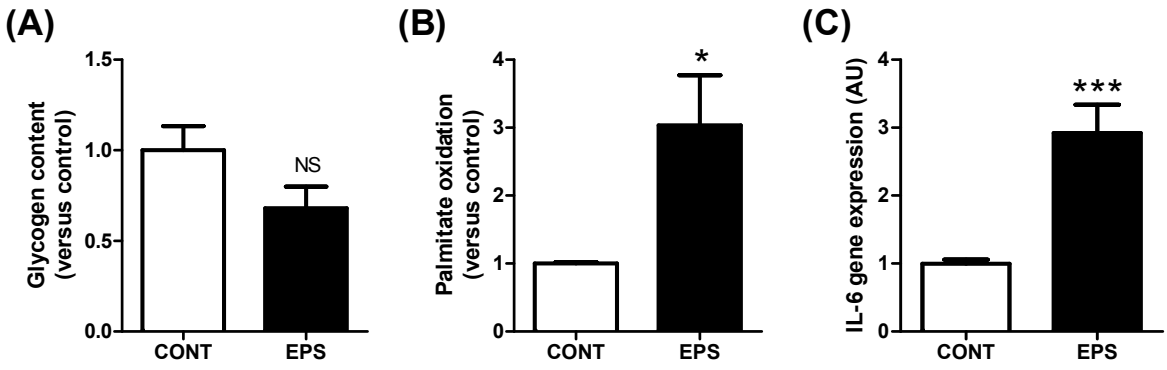
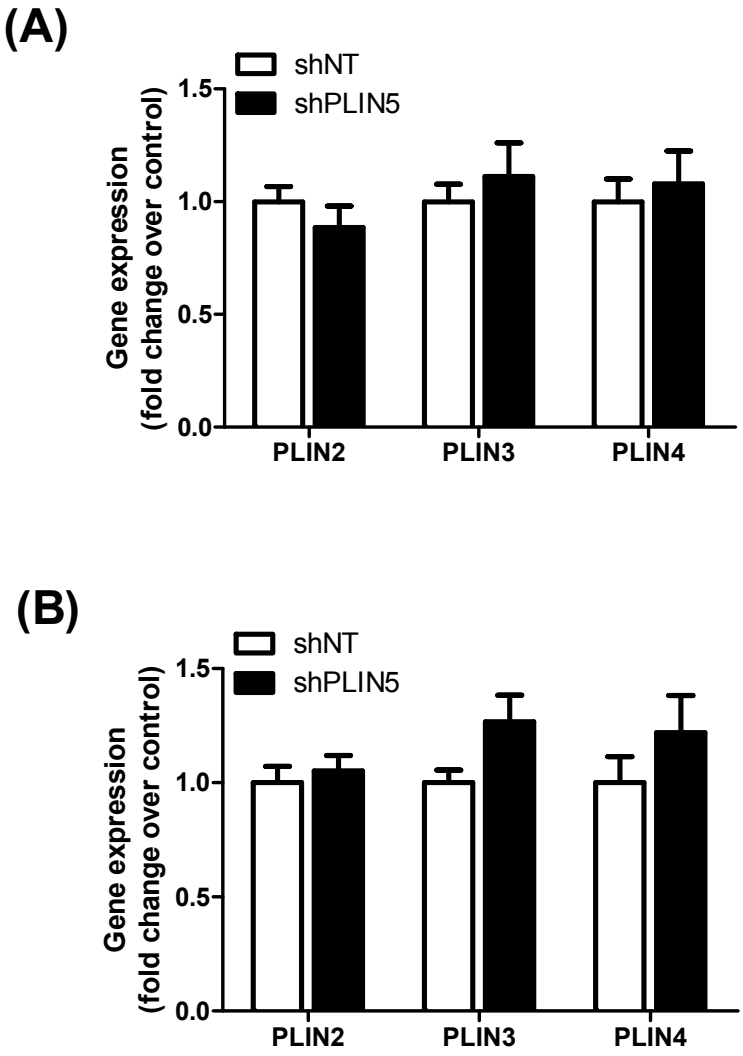


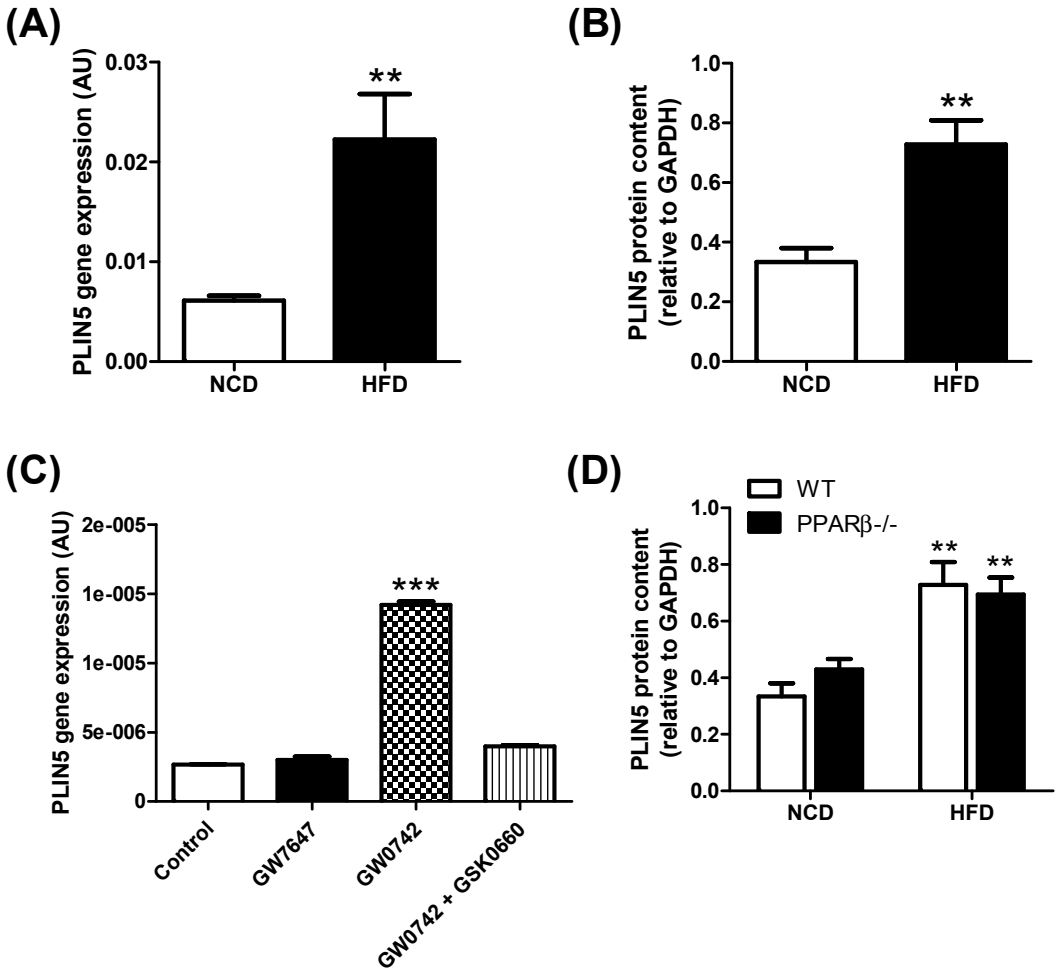
Supplemental Figure S1. PLIN5 gene expression in human native skeletal muscle and cultured myotubes. PLIN5 mRNA levels in human *vastus lateralis* muscle biopsy samples and human primary myotubes (n=9). Average Ct ± SEM are shown on the graph. ***p<0.001 versus hVL.



Supplemental Figure S2. Validation of the electrical pulse stimulation model in human myotubes. (**A**) Total glycogen content (n=4), (**B**) palmitate oxidation (n=4) and (**C**) interleukin-6 (IL-6) gene expression (n=4) were measured in control (CONT) and electrically stimulated (EPS) myotubes for 24 hours. NS : non-significant, *p<0.05, ***p<0.001.



Supplemental Figure S3. PLIN5 knockdown does not induce any compensatory changes in other PLIN isoforms. PLIN2, PLIN3 and PLIN4 gene expression in control (shNT) and PLIN5 knocked down (shPLIN5) *tiabialis anterior* muscles, measured in mice fed either (**A**) normal chow (NCD) or (**B**) high-fat diets for 12 weeks (n=6).



Supplemental Figure S4. PLIN5 is induced by high-fat feeding in mouse skeletal muscle independently of PPARβ activation. PLIN5 (**A**) gene expression and (**B**) protein content were measured in skeletal muscle of mice fed either normal chow (NCD) or high-fat (HFD) diet for 12 weeks (n=7). (**C**) PLIN5 gene expression was measured in myotubes treated for 24 h in absence (control) or presence of selective PPARα agonist GW7647 1 nM, PPARβ agonist GW0742 1nM and PPARβ antagonist GSK0660 500 nM (n=3). (**D**) PLIN5 protein content was measured in skeletal muscle from wild-type (WT) and PPARβ knockout (PPARβ-/-) mice fed either chow (NCD) or high-fat (HFD) diet (n=6). **p<0.01, ***p<0.001 versus control.

8.3.3. Discussion

Les gouttelettes lipidiques jouent un rôle essentiel dans la gestion des IMTG au sein des cellules musculaires, et leur dynamique permet d'adapter le stockage des lipides à leur utilisation, en fonction des besoins énergétiques des cellules. PLIN5 est une protéine de surface des gouttelettes lipidiques fortement exprimée dans les tissus oxydatifs tels que le muscle squelettique, et il a été suggéré que cette protéine serait capable à la fois de protéger la gouttelette de son hydrolyse lorsque les besoins en énergie de la cellule sont faibles, mais également de favoriser la lipolyse lors d'une stimulation β-adrénergique (Mason and Watt, 2015). Cependant, aucune donnée n'est disponible à l'heure actuelle sur cet hypothétique double rôle de PLIN5 dans le muscle squelettique. ***Le but de ce travail a ainsi été de déterminer le rôle fonctionnel de PLIN5 dans le muscle squelettique humain et murin, en conditions basales ainsi que lors d'une augmentation de la demande énergétique (stimulation adrénergique et EPS). Nous avons également évalué le rôle de PLIN5 lors de challenges lipidiques induisant une lipotoxicité, in vitro dans des cultures primaires de cellules musculaires humaines et in vivo chez la souris.***

Dans un premier temps, nous avons observé que l'expression protéique de PLIN5 corrèle avec la capacité oxydative musculaire, chez l'homme et la souris (Figure 25A). De plus, PLIN5 est davantage exprimée dans le muscle de sujets entraînés en endurance que chez des individus sédentaires. De façon intéressante, nous avons constaté que le contenu en PLIN5 musculaire est positivement associé à la sensibilité à l'insuline systémique chez l'homme mesurée lors d'un clamp euglycémique hyperinsulinémique (Figure 25B). Ces résultats sont en accord avec d'autres études ayant montré qu'un entraînement en endurance s'accompagne d'une augmentation de l'expression de PLIN5 ainsi que d'une amélioration de la capacité oxydative et de la sensibilité à l'insuline (Goodpaster *et al.*, 2001; Louche *et al.*, 2013). De façon intéressante, nous avons identifié PLIN5 comme gène cible de PPARβ, acteur clé du métabolisme oxydatif musculaire (Ehrenborg and Krook, 2009), dans des cultures primaires de cellules musculaires humaines, comme cela avait été précédemment montré par l'équipe de K.T. Dalen dans une lignée cellulaire musculaire murine (Bindesboll *et al.*, 2013).

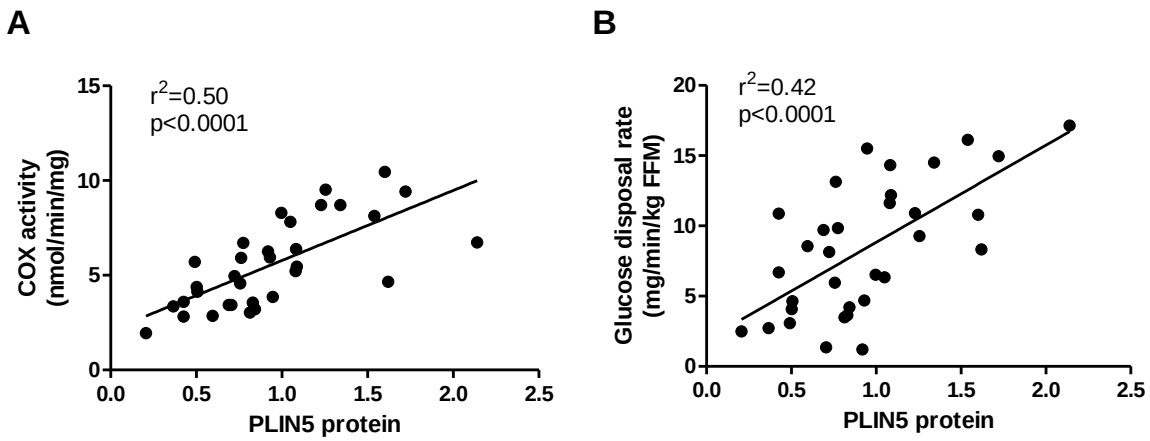


Figure 25. Association entre le contenu musculaire de PLIN5, la capacité oxydative et la sensibilité à l'insuline chez l'homme

Corrélations entre le contenu musculaire de PLIN5 et (A) l'activité de la cytochrome oxidase (COX), (reflétant la capacité oxydative musculaire) et (B) le *glucose disposal rate* mesuré lors d'un clamp hyperinsulinémique euglycémique (reflétant la sensibilité à l'insuline systémique). n=33.

Afin d'étudier le rôle fonctionnel de PLIN5 dans le muscle, nous l'avons dans un deuxième temps surexprimé dans des cultures primaires de cellules musculaires humaines. Nous avons ainsi montré qu'à l'état basal, une surexpression de PLIN5 induit une diminution de la lipolyse musculaire ainsi qu'une plus faible oxydation des AG par rapport aux cellules contrôle. Cette diminution du métabolisme lipidique s'accompagne d'une augmentation du métabolisme glucidique, et ce switch dans l'utilisation des substrats énergétiques pourrait être expliqué par une diminution de l'expression de PDK4, enzyme responsable de l'inhibition de l'oxydation mitochondriale du glucose. Ces résultats sont en accord avec des travaux réalisés chez la souris, montrant qu'une surexpression musculaire de PLIN5 entraîne une augmentation du stockage d'IMTG (Bosma *et al.*, 2013). De façon intéressante, cette étude montre aussi que la surexpression de PLIN5 s'accompagne d'une augmentation de l'expression de gènes impliqués dans le catabolisme des AG et l'oxydation mitochondriale. De plus, deux autres études ont mis en évidence que PLIN5 n'est pas uniquement présente à la surface des gouttelettes lipidiques, mais est aussi retrouvée au niveau des mitochondries (Bosma *et al.*, 2012; Wang *et al.*, 2011). Enfin, il a été reporté, dans des lignées cellulaires autres que le muscle, que PLIN5 est une cible de la protéine kinase A (PKA) et que sa surexpression permettrait d'augmenter l'oxydation des AG en réponse à une stimulation β-adrénergique de la lipolyse (Pollak *et al.*, 2015; Wang *et al.*, 2011). Nous avons

montré dans cette étude que, alors que PLIN5 diminue l'oxydation des AG en conditions basales dans des cultures primaires de cellules musculaires humaines, sa surexpression favorise l'oxydation des AG lorsque les besoins énergétiques de la cellule augmentent, soit suite à une stimulation β-adrénergique (Figure 26A), soit en réponse à la contraction des cellules musculaires (Figure 26B). Ces résultats suggèrent que PLIN5 pourrait permettre de créer un lien entre les gouttelettes lipidiques et les mitochondries, permettant ainsi un adressage direct des AG à ces dernières. Ce couplage pourrait contribuer à protéger la cellule d'une accumulation d'espèces lipotoxiques entraînant une altération de la sensibilité à l'insuline. De façon intéressante, nous avons constaté que la surexpression de PLIN5 protège les cellules de l'accumulation d'intermédiaires lipotoxiques et de l'altération de la sensibilité à l'insuline induite par un challenge lipidique.

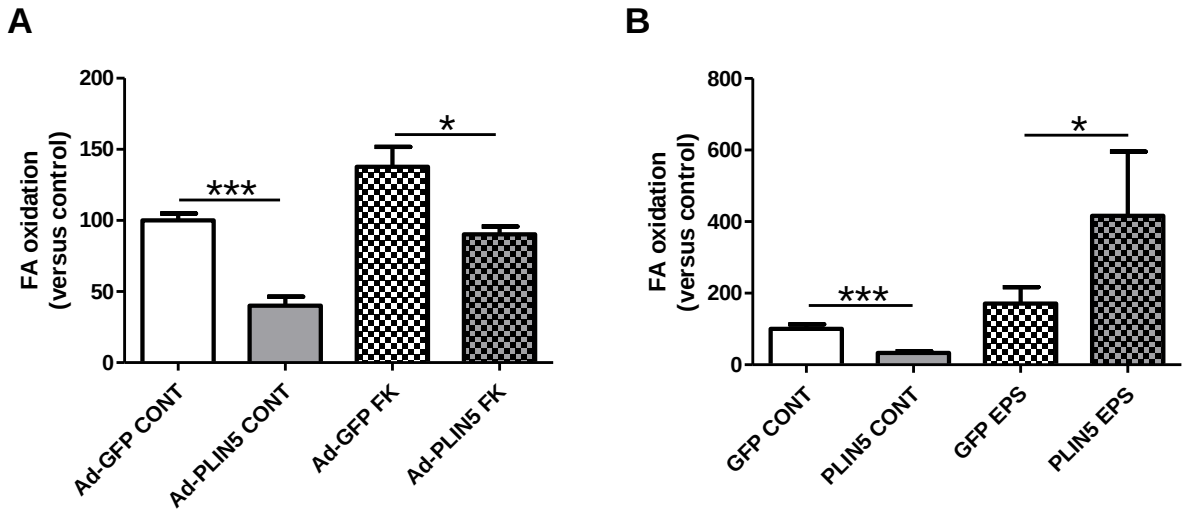


Figure 26. Une surexpression de PLIN5 favorise l'oxydation des AG lors d'une augmentation de la demande énergétique

Mesure de l'oxydation d'AG radiomarqués dans des myotubes contrôles (Ad-GFP) ou surexprimant PLIN5 (Ad-PLIN5), en condition basale (CONT) ou suite à (A) une stimulation β-adrénergique de la lipolyse induite par un traitement avec de la forskoline (FK) et (B) lors de stimulations électriques des myotubes induisant leur contraction (EPS). n=6. *p<0.05, p<0.001 vs Ad-GFP.

Enfin, nous avons évalué le rôle physiologique de PLIN5 dans le muscle squelettique *in vivo* chez la souris. Considérant que PLIN5 est fortement exprimée dans le muscle natif, et que sa surexpression ne permet pas d'observer, hormis l'accumulation d'IMTG, de changements notables dans la régulation du métabolisme lipidique (Bosma *et al.*, 2013), nous avons décidé d'étudier les conséquences de son

invalidation dans le muscle squelettique. Nous avons tout d'abord constaté que, en accord avec les résultats obtenus *in vitro* en conditions basales, l'invalidation musculaire de PLIN5 entraîne une augmentation de l'oxydation des AG, probablement via un effet de flux, en permettant un meilleur accès des lipases aux IMTG contenus dans les gouttelettes lipidiques. De plus, en accord avec l'association observée chez l'homme entre l'expression musculaire de PLIN5 et la sensibilité à l'insuline, nous mettons en évidence qu'une invalidation de PLIN5 dans le muscle chez la souris entraîne une diminution du transport musculaire de glucose induit par l'insuline. Ces résultats sont cohérents avec le phénotype insulino-résistant observé chez les souris présentant une délétion systémique de PLIN5 (Mason *et al.*, 2014).

Afin d'établir le rôle de PLIN5 dans un contexte d'insulino-résistance, nous l'avons invalidé dans le muscle squelettique de souris nourries avec un régime riche en graisses. De façon surprenante, l'invalidation de PLIN5 dans ce contexte induit une augmentation du transport musculaire de glucose stimulé par l'insuline (Figure 27A). Il est important de noter que de précédents travaux ont démontré qu'un régime riche en graisses s'accompagne d'une augmentation compensatoire de la capacité oxydative musculaire, qui n'est toutefois pas suffisante pour prévenir l'accumulation d'IMTG et d'espèces lipotoxiques si cette situation perdure (Hancock *et al.*, 2008; Oakes *et al.*, 2013). Nous pouvons donc émettre l'hypothèse que diminuer l'expression de PLIN5 dans ce contexte permettrait de faciliter l'hydrolyse des IMTG et l'oxydation des AG, protégeant ainsi la cellule d'une pression lipotoxique. Ces résultats sont supportés par le fait que les muscles invalidés pour PLIN5 sont partiellement protégés de l'accumulation de céramides induite par la prise d'un régime gras (Figure 27B). Ces résultats sont en accord avec une autre étude ayant montré que les souris délétées pour PLIN5 présentent une amélioration de la tolérance au glucose et une tendance à l'augmentation sa clairance tissulaire lorsqu'elles sont nourries avec un régime gras (Mason *et al.*, 2014).

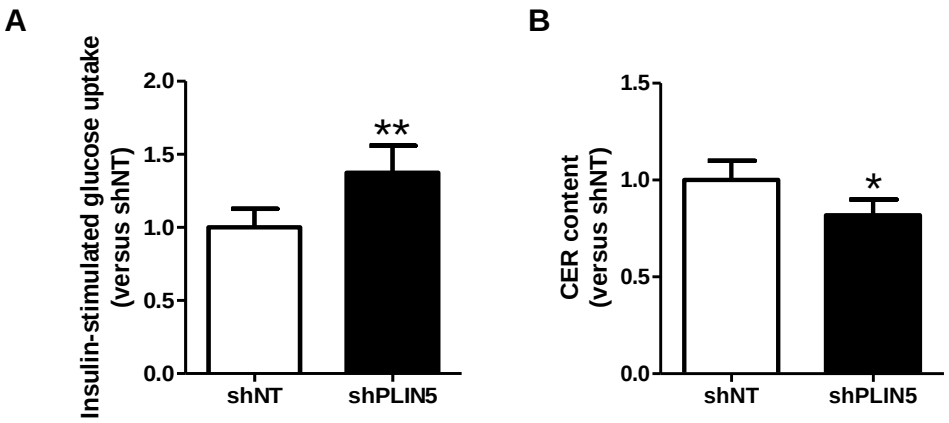


Figure 27. L'invalidation de PLIN5 dans le muscle squelettique *in vivo* induit une amélioration de la sensibilité à l'insuline musculaire dans un contexte d'obésité induite par la prise d'un régime hyperlipidique

(A) Mesure du transport musculaire de glucose *in vivo* en réponse à l'insuline, dans des muscles contrôle (shNT) ou invalidés pour PLIN5 (shPLIN5). (B) Quantification du contenu musculaire en céramides (CER) dans des muscles contrôle (shNT) ou invalidés pour PLIN5 (shPLIN5). n=7. *p<0.05, **p<0.01 vs shNT.

En conclusion, ces données démontrent pour la première fois que PLIN5 joue un double rôle dans le muscle squelettique, favorisant le stockage d'IMTG et protégeant les cellules d'une accumulation intracellulaire d'intermédiaires lipotoxiques à l'état basal, et facilitant la lipolyse et l'oxydation des AG lorsque les besoins énergétiques des cellules augmentent. Enfin, nous mettons en évidence un rôle clé de PLIN5 dans le contrôle de la dynamique des gouttelettes lipidiques et de la sensibilité à l'insuline musculaire (Figure 28).

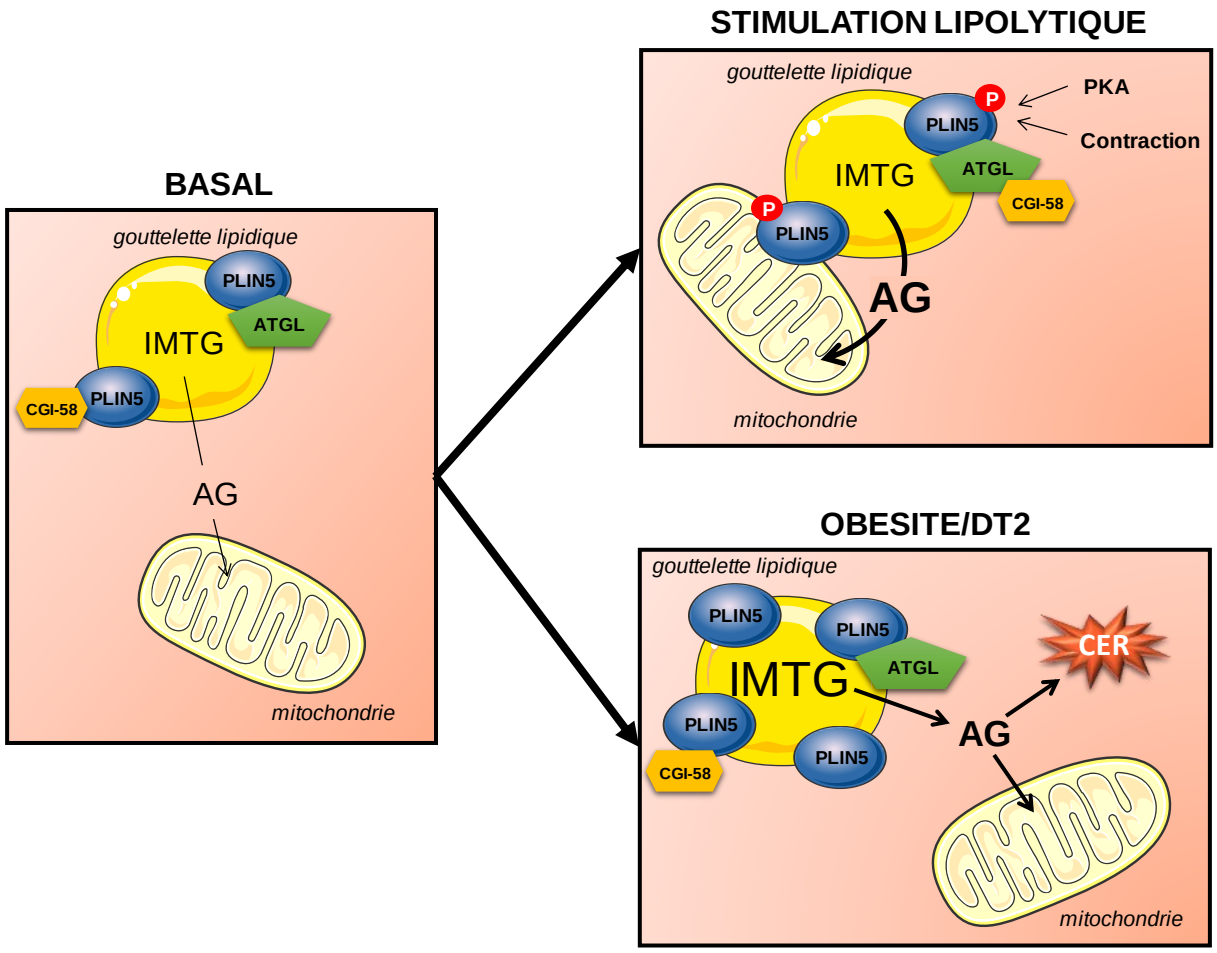


Figure 28. Modèle représentant le contrôle de la lipolyse musculaire par PLIN5

A l'état basal, PLIN5 serait capable de lier indépendamment l'*adipose triglyceride lipase* (ATGL) et son co-activateur *comparative gene identification-58* (CGI-58), empêchant ainsi leur intéraction et une activation maximale de l'ATGL. Lors de la contraction, ou suite à une activation de la protéine kinase A (PKA), la phosphorylation de PLIN5 induirait la libération de CGI-58 favorisant ainsi son intéraction avec l'ATGL et une augmentation de l'hydrolyse des IMTG. PLIN5 pourrait aussi permettre de favoriser l'adressage des acides gras (AG) des gouttelettes lipidiques vers les mitochondries, en créant un lien physique entre ces deux organelles. Lors du développement de l'obésité et du diabète de type 2 (DT2), l'expression de PLIN5 est augmentée conduisant à une accumulation d'IMTG et d'espèces lipotoxiques telles que les céramides (CER), impliquées dans l'inhibition de la signalisation insulinique.

9. CONCLUSION ET PERSPECTIVES

Ce travail de thèse a permis d'une part de mettre en évidence un lien causal entre accumulation d'IMAT et insulino-résistance musculaire chez l'homme, et d'autre part d'améliorer la compréhension de la régulation de la lipolyse musculaire, et notamment de montrer que G0S2 et PLIN5, deux protéines de la gouttelette lipidique, sont au centre du contrôle de l'homéostasie lipidique et du maintien de la sensibilité à l'insuline au sein du muscle squelettique.

Nos résultats ont permis de mettre en évidence l'existence d'un dialogue entre FAP différenciés en adipocytes et fibres musculaires dans le muscle de sujets obèses *in vitro*. Nous pouvons émettre l'hypothèse que, comme cela a été observé pour les cellules satellites (Bourlier *et al.*, 2013; Ukropcova *et al.*, 2005), les FAP puissent retenir les caractéristiques métaboliques du donneur dont elles sont issues. Il serait ainsi intéressant d'évaluer l'impact de milieux conditionnés de FAP différenciés en adipocytes issus de sujets présentant des statuts métaboliques différents (*i.e.* sujets sains, obèses, diabétiques de type 2) ou lors d'études cliniques interventionnelles (*i.e.* entraînement physique et/ou restriction calorique) sur la sensibilité à l'insuline des fibres musculaires. Par ailleurs, sachant qu'une accumulation d'IMAT est observée chez des sujets âgés et est associée à une altération métabolique et fonctionnelle du muscle (Addison *et al.*, 2014), il serait également intéressant de comparer le sécrétome de FAP différenciés en adipocytes issus de sujets jeunes et de sujets âgés.

Une autre question que soulève ce travail est la nature des facteurs impliqués dans cette communication entre IMAT et fibres musculaires. Il a été montré, par des expériences de co-culture, que les adipocytes différenciés à partir de progéniteurs issus de la fraction SVF de tissu adipeux de la glande mammaire entraînent une altération de la signalisation insulinique des cellules musculaires humaines, associée à une diminution de la translocation de GLUT4 à la membrane plasmique (Eckardt *et al.*, 2008). Il est toutefois important de noter que, alors que le TNF-α est capable de reproduire la diminution de phosphorylation d'IRS1 et d'Akt observée lors des

expériences de co-culture, ce facteur inflammatoire n'est pas retrouvé dans les milieux conditionnés d'adipocytes issus de la glande mammaire (Dietze *et al.*, 2002), suggérant que d'autres adipo/cytokines seraient impliquées dans ce processus (Lee *et al.*, 2009; Sell *et al.*, 2006). De plus, il est maintenant communément admis que chaque dépôt adipeux possède des propriétés (*i.e.* métaboliques, phénotypiques, sécrétoires) différentes (Lee *et al.*, 2013; Tchkonia *et al.*, 2013), et il a été montré que l'IMAT présente des similarités mais aussi de fortes différences avec les autres dépôts adipeux (Arrighi *et al.*, 2015; Gardan *et al.*, 2006; Gondret *et al.*, 2008). Il sera donc important, dans de futurs travaux de recherche, de caractériser spécifiquement le sécrétome des adipocytes intramusculaires obtenus *in vitro*, afin d'identifier de potentiels candidats responsables de l'altération métabolique des fibres musculaires, et de ne pas transposer les résultats obtenus avec des adipocytes issus de tissu adipeux sous-cutané, viscéral ou mammaire.

Par ailleurs, l'origine cellulaire de l'émergence des adipocytes au sein du muscle reste débattue au sein de la communauté scientifique. Nos résultats montrent que les FAP présents dans le muscle squelettique constituent une population cellulaire différente des cellules satellites. Cependant, d'autres cellules progénitrices pourraient être à l'origine du développement de l'IMAT. Sachant que l'obésité est caractérisée par un développement important du tissu adipeux et s'accompagne d'une accumulation d'IMAT (Boettcher *et al.*, 2009; Gallagher *et al.*, 2009; Goodpaster *et al.*, 2000), et qu'il a été montré que le tissu adipeux contient des cellules capables de rejoindre la circulation et de migrer dans d'autres organes (Forcales, 2015; Gil-Ortega *et al.*, 2013; Liu *et al.*, 2007b; Miura *et al.*, 2008; Mizuno, 2010), il est possible d'imaginer que des progéniteurs adipocytaires puissent quitter ce tissu et rejoindre le muscle squelettique, contribuant ainsi à l'accumulation d'IMAT dans cet organe dans un contexte d'obésité. Il serait ainsi intéressant d'identifier des marqueurs spécifiquement exprimés dans les progéniteurs adipocytaires du tissu adipeux blanc et absents des FAP, et inversement. Cela permettrait notamment de créer un modèle animal dans lequel la GFP serait exprimée spécifiquement dans les progéniteurs adipocytaires du tissu adipeux (*i.e.* ASC), et de nourrir ces animaux avec un régime gras afin de les rendre obèses. Si des cellules exprimant la GFP sont retrouvées dans le muscle squelettique de ces animaux, cela signifierait que

l'accumulation d'IMAT observée avec l'obésité est au moins en partie due à une fuite des progéniteurs adipocytaires du tissu adipeux.

Enfin, nous ne pouvons pas exclure l'hypothèse que l'accumulation d'IMAT observée avec l'obésité puisse être un mécanisme adaptatif lors de périodes de surcharge lipidique, afin de tamponner l'excès toxique de lipides, évitant ainsi leur accumulation au sein des cellules musculaires. Cependant, si cette situation perdure, l'IMAT présent en excès pourrait sécréter des facteurs qui, de façon paracrine, contribueraient à l'altération de la sensibilité à l'insuline des fibres musculaires. Il serait ainsi intéressant de dépléter sélectivement les FAP au sein du muscle squelettique, par exemple en créant un modèle animal exprimant le récepteur de la toxine diphtérique (Buch *et al.*, 2005) sous le contrôle du promoteur d'un gène spécifiquement exprimé dans les FAP, non identifié à l'heure actuelle, et suivre le développement précoce de l'insulino-résistance induite par l'obésité chez ces animaux. En accord avec l'hypothèse d'un rôle causal de l'IMAT dans le développement de l'insulino-résistance musculaire, des résultats très préliminaires de notre laboratoire ont montré que, suite à une injection intramusculaire de glycérol chez la souris, une accumulation d'IMAT est observée et est associée à une tendance à la diminution du transport de glucose stimulé par l'insuline dans le muscle injecté.

Nous avons d'autre part démontré un rôle clé de G0S2 et PLIN5, deux protéines présentes à la surface des gouttelettes lipidiques, dans la régulation de la lipolyse musculaire. Afin de compléter ces travaux, il serait intéressant de développer des modèles animaux présentant une délétion spécifique de ces protéines au sein du muscle squelettique. Cela permettrait d'enrichir les connaissances actuelles sur la contribution spécifique de dérégulations de la dynamique des gouttelettes lipidiques musculaires dans le développement d'une insulino-résistance systémique.

D'autre part, nous avons constaté une diminution de l'action de l'insuline dans le muscle squelettique lorsque PLIN5 est invalidé dans cet organe, qui n'est cependant pas associée à une accumulation intracellulaire de céramides. De façon intéressante, il a été montré que des dérégulations du métabolisme lipidique peuvent générer une inflammation métabolique chronique de bas grade, appelée

« metaflammation » (Hotamisligil, 2006). Certaines espèces lipidiques, comme les AG saturés ou le cholestérol, entraînent une augmentation du stress du réticulum endoplasmique, indépendamment du contenu en céramides (Wei *et al.*, 2006). Ce dernier entraîne alors l'activation de kinases inflammatoires telles que IKK, JNK et PKR (Nakamura *et al.*, 2010; Ozcan *et al.*, 2004), responsables d'une altération de la signalisation insulinique, notamment au niveau d'IRS1 et Akt (Erikci Ertunc and Hotamisligil, 2016; Flamment *et al.*, 2012; Hage Hassan *et al.*, 2016). Mesurer l'activation de ces kinases pourrait ainsi permettre de mieux comprendre les mécanismes à l'origine de l'altération de la sensibilité à l'insuline dans les muscles invalidés pour PLIN5.

Par ailleurs, il est maintenant communément admis que les gouttelettes lipidiques sont des organites complexes, possédant un protéome spécifique et capables d'interagir avec différentes structures intracellulaires (Gao and Goodman, 2015). Comprendre la façon dont le protéome des gouttelettes lipidiques pourrait influencer leur dynamique pourrait conduire à l'identification de nouvelles protéines jouant un rôle important dans le métabolisme lipidique musculaire. De plus, il semble que différentes populations de gouttelettes lipidiques, présentant des différences de protéome, de taille et de localisation sub-cellulaire, co-existent au sein d'une même cellule et exercent des fonctions spécifiques (Beller *et al.*, 2006; Wolins *et al.*, 2003). Il a par exemple été suggéré que les gouttelettes de petite taille, ayant une surface de contact avec les lipases cytosoliques relativement plus grande que les gouttelettes de taille plus importante, auraient une capacité accrue à mobiliser les lipides qu'elles contiennent (Bosma, 2016). En accord avec cette hypothèse, il a été décrit que la pratique d'un entraînement en endurance est associée à une augmentation de la quantité d'IMTG stockés dans un plus grand nombre de gouttelettes de petite taille (plutôt qu'à une augmentation du volume des gouttelettes préexistantes) et que cela est associé à une augmentation de la capacité des cellules musculaires à oxyder les lipides (Shepherd *et al.*, 2013; Tarnopolsky *et al.*, 2007). De plus, il a été mis en évidence, par microscopie confocale dans des adipocytes en culture, une expression différentielle des isoformes de périlipines selon la taille des gouttelettes. Ainsi, les périlipines 3 et 4 sont exprimées sur les gouttelettes naissantes, en périphérie des cellules. La périlipine 2 est majoritairement présente sur les gouttelettes de taille intermédiaire, les plus grosses gouttelettes sont

quant à elles présentes au centre de la cellule et la périlipine 1 est l'isoforme le plus exprimé à leur surface (Wolins *et al.*, 2006a). Il serait intéressant de déterminer, par une approche d'imagerie confocale, si PLIN5 est davantage présente sur des gouttelettes lipidiques de petite ou grande taille au sein des cellules musculaires, en condition basale, et si une relocalisation a lieu suite à une stimulation lipolytique ou à un entraînement en endurance chez l'homme.

Il a également été décrit que, au-delà de la modification des protéines recrutées à la surface des gouttelettes, une stimulation lipolytique répétée induite par la pratique d'un entrainement en endurance entraîne une relocalisation de ces gouttelettes de l'espace subsarcolemmal vers l'espace intermyofibrillaire, ainsi qu'une augmentation de leur surface de contact avec les mitochondries (Devries *et al.*, 2013). Par ailleurs, il a été mis en évidence que Arf1/COPI, complexe protéique impliqué dans les mouvements vésiculaires, permet de créer des liens physiques entre le réticulum endoplasmique et les gouttelettes lipidiques afin d'adresser les enzymes lipogéniques et lipolytiques à la surface des gouttelettes (Wilfling *et al.*, 2014). Afin de mieux comprendre ces mécanismes, et d'étudier leur implication potentielle dans la gestion des réserves lipidiques dans différents contextes métaboliques, il serait intéressant de marquer, par une approche d'immunofluorescence, les protéines des gouttelettes lipidiques dans des cellules musculaires issues de donneurs présentant différents statuts métaboliques, et de suivre en temps réel, par microscopie confocale, la dynamique des différentes populations de gouttelettes ainsi identifiées suite à une stimulation lipolytique β-adrénergique ou lors de la contraction de ces cellules *in vitro*.

Enfin, une élégante étude de l'équipe de M.D. Jensen a mis en évidence chez l'homme que les AG circulants qui pénètrent dans les cellules musculaires transitent en grande partie par les IMTG contenus dans les gouttelettes lipidiques avant d'être oxydés par les mitochondries (Dagenais *et al.*, 1976; Kanaley *et al.*, 2009). Cette observation confère un rôle central aux gouttelettes lipidiques dans la gestion du métabolisme lipidique musculaire. De façon contradictoire, il a également été montré dans d'autres études qu'une grande partie des AG entrant dans les cellules musculaires est directement oxydée (Kiens, 2006; Roepstorff *et al.*, 2002). Cependant, ces études ont été réalisées lors de la pratique d'un exercice physique, et ont montré que le contenu en IMTG n'était pas modifié pendant l'exercice, et

diminuait en post-exercice. Une explication pourrait être que le contenu en IMTG n'est pas modifié car l'exercice induit une augmentation de l'incorporation d'AG plasmatique au sein des IMTG (Guo *et al.*, 2000). Le couplage entre cette estérification d'AG et la lipolyse des IMTG pourrait être à l'origine du maintien de la quantité d'IMTG à l'exercice. De façon intéressante, il a été montré qu'une incorporation maximale des AG plasmatiques dans les pools d'IMTG est observée lors de la pratique d'un exercice à une intensité sub-maximale (Guo *et al.*, 2000), et il a été suggéré que ceci puisse être dû à une plus grande proportion de fibres inactives lors de ce type d'exercice (Jordy and Kiens, 2014). Afin de mieux caractériser ces mécanismes, il serait intéressant de mesurer, à l'aide d'AG radio-marqués, la part d'AG directement oxydés de celle qui transite par les gouttelettes lipidiques dans des cultures primaires de cellules musculaires humaines au repos, ou suite à des contractions induites par des stimulations électriques de différentes intensités.

De façon plus générale, étant donné que les niveaux d'intermédiaires lipidiques lipotoxiques sont augmentés chez les sujets diabétiques et jouent un rôle causal dans le développement de l'insulino-résistance, il apparait donc que cibler les lipides musculaires dans le cadre du diabète de type 2 présente un intérêt thérapeutique important. Il sera toutefois difficile, dans l'état actuel des connaissances, de cibler spécifiquement le muscle squelettique afin de délivrer des composés modulant l'expression des enzymes lipolytiques. Les progrès importants dans le domaine de la thérapie génique pourront peut-être permettre dans les prochaines années de moduler spécifiquement l'expression de certains gènes au sein d'organes qu'il est compliqué de cibler autrement, comme c'est le cas pour le muscle squelettique.

En résumé, l'ensemble de ce travail de thèse a permis de contribuer à l'enrichissement des connaissances sur le rôle des lipides musculaires dans la régulation du métabolisme énergétique et le maintien de la sensibilité à l'insuline au sein du muscle squelettique (Figure 29). Cependant, de nombreux points restent encore à éclaircir et pourront faire l'objet de futurs travaux de recherche dans les prochaines années.

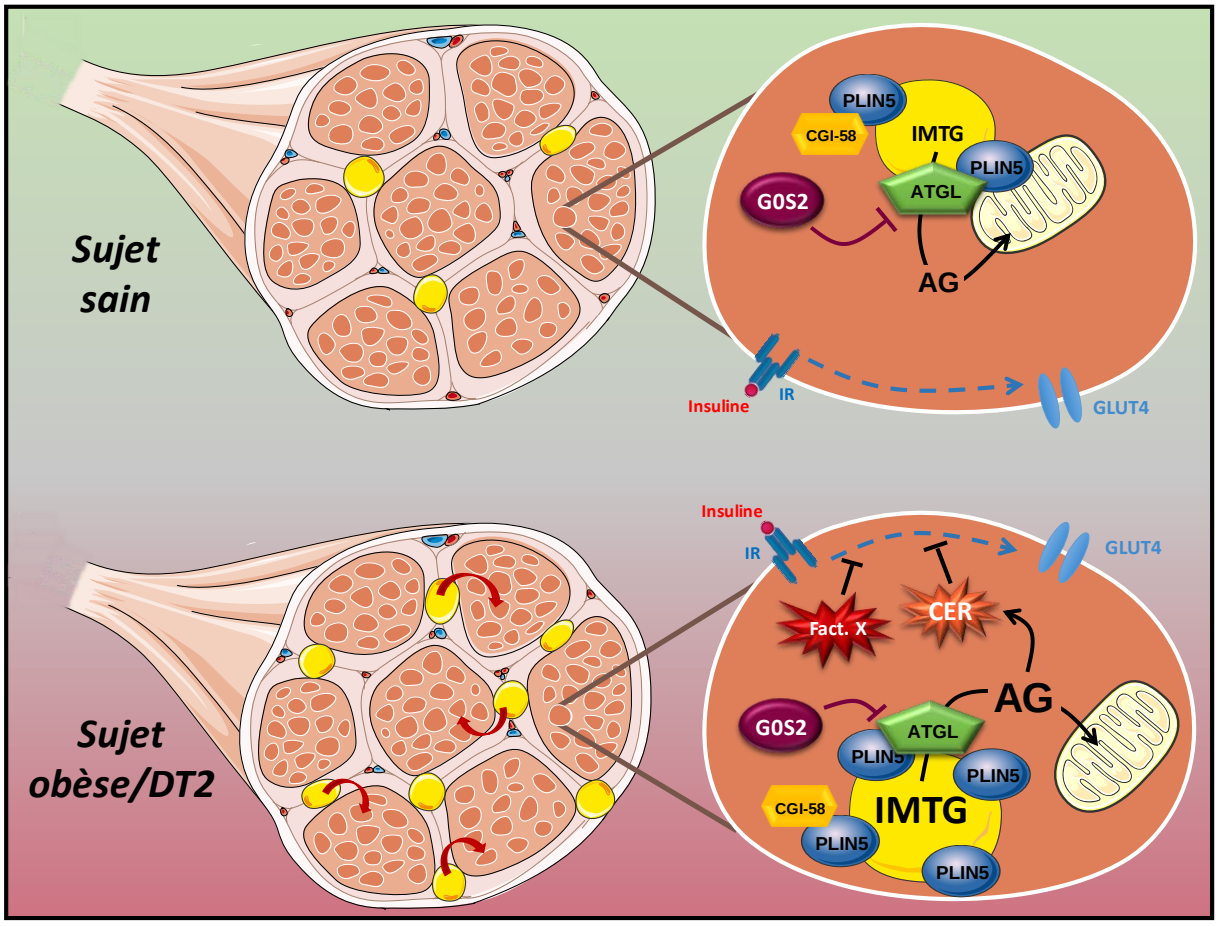


Figure 29. Modèle représentant le rôle des lipides musculaires dans le développement de l'insulino-résistance associée à l'obésité

L'obésité et le diabète de type 2 (DT2) s'accompagnent d'une accumulation de triacylglycérols intramyocellulaires (IMTG) et d'adipocytes intramusculaires. L'augmentation du flux lipolytique intramyocellulaire, dans le contrôle duquel G0S2 et PLIN5 jouent un rôle central, conduit à une accumulation d'intermédiaires lipotoxiques comme les céramides (CER) capables d'inhiber la voie de signalisation de l'insuline. Par ailleurs, les progéniteurs fibro/adipogéniques différenciés en adipocytes sont capables de sécréter des facteurs, qui n'ont pas été identifiés à l'heure actuelle (flèches rouges, « fact. X ») responsables d'une altération de la signalisation insulinique au sein des fibres musculaires. IR : récepteur à l'insuline ; GLUT4 : *glucose transporter 4* ; CGI-58 : *comparative gene identification-58* ; G0S2 : *G0/G1 switch gene 2* ; PLIN5 : périlipine 5 ; AG : acides gras

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ANNEXES

PUBLICATION 4 : ROLE DES LIPIDES INTRAMYOCELLULAIRES DANS LES MALADIES METABOLIQUES

Intramyocellular fat storage in metabolic diseases

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Abstract: Over the past decades, obesity and its metabolic co-morbidities such as type 2 diabetes (T2D) developed to reach an endemic scale. However, the mechanisms leading to the development of T2D are still poorly understood. One main predictor for T2D seems to be lipid accumulation in "non-adipose" tissues, best known as ectopic lipid storage. A growing body of data suggests that these lipids may play a role in impairing insulin action in metabolic tissues, such as liver and skeletal muscle. This review aims to discuss recent literature linking ectopic lipid storage and insulin resistance, with emphasis on lipid deposition in skeletal muscle. The link between skeletal muscle lipid content and insulin sensitivity, as well as the mechanisms of lipid-induced insulin resistance and potential therapeutic strategies to alleviate lipotoxic lipid pressure in skeletal muscle will be discussed.

Keywords: ectopic lipids; IMTG; lipotoxicity; skeletal muscle; type 2 diabetes.

Introduction

Nowadays, obesity has become one of the most prevalent disease worldwide, leading to the development of metabolic and cardiovascular pathologies [1]. Obesity is currently the strongest risk factor known for type 2 diabetes (T2D). T2D is characterized by a fasting hyperglycemia that is due to an impaired action of insulin on insulin sensitive tissues (i.e. adipose tissue, liver and skeletal muscle), the so-called insulin resistance [2, 3]. In the last decades, at least two mutually not exclusive hypotheses have emerged linking obesity to insulin resistance as recently reviewed by Samuel and Shulman [4]. One prevailing hypothesis is lipotoxicity caused by lipid overflow out of adipose tissue and ectopic lipid storage in lean tissues. This situation typically occurs when excess dietary lipids cannot anymore be appropriately stored into adipose tissue. Thus an enhanced expansion of fat mass is thought to protect against metabolic disturbances and to convey a healthy obese profile [5]. As a consequence, excess lipid accumulation in pancreas causes beta cell dysfunction. Some data suggest that, in a context of hyperglycemia, fatty acids can induce beta-cell death by apoptosis, a process called glucolipotoxicity [6].

In liver, ectopic lipid accumulation is referred as nonalcoholic fatty liver disease (NAFLD). Increased hepatocellular lipid content correlates negatively with both whole body and hepatic insulin sensitivity, leading to impaired suppression of endogenous glucose production and decreased hepatic glycogen synthesis during euglycemic hyperinsulinemic clamp [7]. When T2D patients are subjected to a hypocaloric diet, a strong decrease of 85% of hepatic lipid content is observed, associated with a normalization of hepatic insulin sensitivity as well as decreased hyperglycemia and hepatic glucose production. Importantly, these changes occur without any modifications of skeletal muscle lipid content, suggesting that the reduction of hepatic lipids per se could improve insulin sensitivity [8].

Numerous studies have linked ectopic lipid accumulation in skeletal muscle and insulin resistance [9–13]. Skeletal muscle plays a major role in whole body glucose homeostasis, as it is responsible for 80% of glucose disposal in response to insulin in the postprandial phase [14, 15]. Thus, as this organ acts as a metabolic sink for glucose, it is easy to figure out that impaired insulin action in muscle will lead to whole body insulin resistance and further development of T2D.

The purpose of this review was to summarize current understanding of how lipids emerge in skeletal muscle and to what extent they can contribute to muscle and whole body insulin resistance.

Skeletal muscle ectopic lipids and insulin resistance

Lipids can be found under two different forms in skeletal muscle: adipose cells, located between muscle fibers (i.e.

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intramuscular adipose tissue), and lipid droplets inside muscle fibers (i.e. intramyocellular triglycerides) [16, 17] (Figure 1).

Intramuscular adipose tissue

Intramuscular fat is made of adipose cells (i.e. adipocytes) located between muscle fibers and muscle groups. Different studies in rodents and humans report a positive correlation between intramuscular adipose tissue (IMAT) and insulin resistance [18–20], and even when body mass index (BMI) is accounted for, it remains a strong predictor of fasting glucose and insulin levels [21].

However, it is still unclear if these adipocytes emerge from resident adipogenic progenitors or from satellite cells in skeletal muscle [16, 22]. Recent data from our group suggest that resident adipogenic progenitors, with potential to differentiate into functional adipocytes in vitro, are present in human skeletal muscle. We suggest that the proximity of these adipocytes to muscle fibers may impair muscle insulin action in a paracrine manner, and thus impact on whole body insulin sensitivity. On the other hand, one could speculate that these adipogenic progenitors differentiate into mature adipocytes as an adaptive mechanism during periods of lipid overload, as a buffer system to prevent lipid accumulation within myofibers. Future studies should investigate if IMAT can cause muscle dysfunction.

Intramyocellular triglycerides

In muscle fibers, lipids are stored within lipid droplets (LD) as triacylglycerols (TAG). Intramyocellular triglycerides (IMTG) constitute an important energy fuel source during muscle contraction [23, 24]. Indeed, elegant studies from Jensen's group demonstrated that plasma fatty acids are first esterified into IMTG pools before being oxidized, both at rest [25, 26] and during exercise [27]. They further demonstrated that during submaximal exercise IMTG turnover is high, without changes in the IMTG pool size. However, other studies report that 60%–100% of radiolabeled oleate or palmitate taken up in skeletal muscle was directly oxidized [24, 28]. Such discrepancies may be due to differences in experimental protocol and/or training status of the subjects, which really differs from one study to another [23].

Importantly, maximal fatty acid oxidation in skeletal muscle is observed at moderate exercise intensities between 45 and 65% of maximal oxygen uptake depending on the type of subjects [24].

However, increased IMTG content is also found in obese and T2D people [10], and studies in lean healthy individuals report IMTG content as a stronger predictor

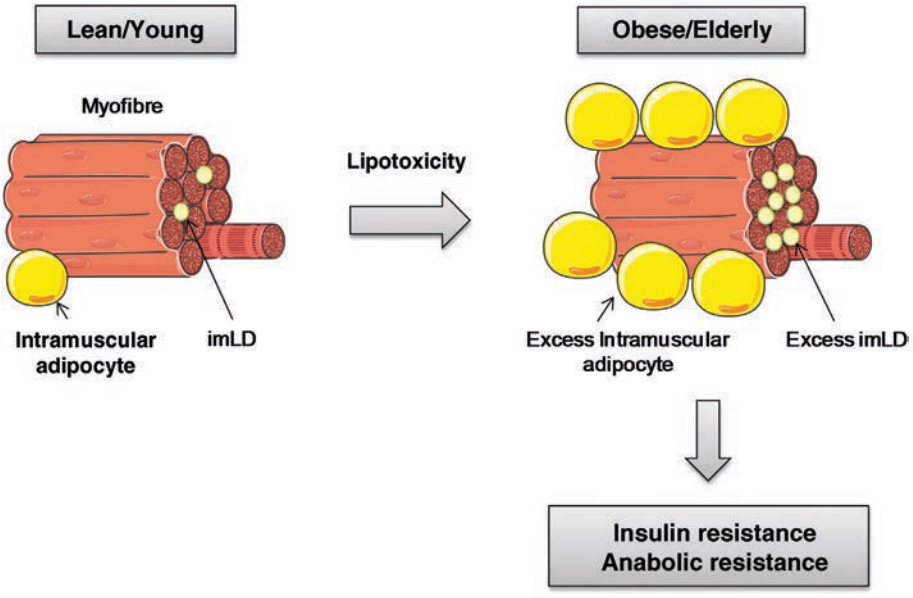

Figure 1: Model linking obesity, muscle lipids and insulin resistance.
This cartoon illustrates that in young and lean subjects, a physiological amount of lipids is present in skeletal muscle. These lipids are stored in intramuscular adipocytes located between muscle fibers and in intramyocellular lipid droplets (imLD) within muscle fibers. When obesity develops, and this is also observed with aging, these lipids are stored in excess in skeletal muscle, leading to lipotoxicity responsible for insulin and anabolic resistance.

of muscle insulin resistance than circulating fatty acids [29]. However, these studies did not account for the sedentary or active status of the subjects. In sedentary individuals, a strong inverse relationship has been reported between IMTG content and insulin sensitivity, independently of body mass index [30]. However, this relationship has proven much more complex than initially thought. Indeed, Goodpaster and colleagues have demonstrated that the skeletal muscle of endurance-trained athletes is highly insulin sensitive despite having an elevated IMTG content [31]. This could be explained by a more efficient lipid handling in skeletal muscle of athletes, allowing an optimal coupling between lipid storage and utilization to avoid lipotoxic insults. Interestingly, a close physical association between LD and mitochondria has been reported in active versus sedentary subjects, as well as a higher turnover of the IMTG pool [32–36].

Lipotoxic lipids

In the last few years, several studies aimed to identify the molecular actors responsible for the impairment of insulin signaling in skeletal muscle. It is now well established that IMTG per se do not cause insulin resistance, and several evidence dissociate TAG content from insulin resistance in humans [37, 38]. These observations raised the question of which molecular triggers could link increased IMTG content and insulin resistance in T2D subjects. Several lipid species emerged as potential candidates and in particular diacylglycerols (DAG) and ceramides (CER) [39, 40]. Indeed, increased DAG and CER content in skeletal muscle has been associated with insulin resistance in rodents [41, 42] and humans [43, 44]. A study from Goodpaster and colleagues demonstrated that diet-induced weight loss and exercise improved insulin resistance while reducing muscle DAG and CER content [45]. However, controversies exist related to the role of these lipid metabolites in impairing muscle insulin signalling, as some studies describe no change in muscle DAG and CER content associated with insulin resistance [17, 46–48], although data are not consistent [17, 49]. Taken together, these studies reveal that it seems that subcellular localization and composition of DAG [50] and CER, as well as their stereospecificity play an important role in their ability to impede insulin action, and needs to be further investigated.

Diacylglycerols pools

In rats, intralipid/heparin induces a strong increase of plasma fatty acids, and starts to reduce insulin sensitivity 3h after the beginning of infusion concomitant with the accumulation of DAG, impaired insulin signalling and glucose uptake in skeletal muscle. Of importance, insulin resistance occurred without any changes in IMTG content [40, 51]. DAGs act as signalling molecules, and are allosteric activators of protein kinase C (PKC). It has been shown that DAG-induced activation of novel PKC isoforms, and particularly PKCθ and PKCε in skeletal muscle, induces serine phosphorylation of insulin receptor substrate 1 (IRS1), thereby inhibiting insulin signalling [40, 52, 53].

Ceramides pools

It is interesting to note that mice fed for 12 weeks with a high-fat diet are protected from glucose intolerance if they are treated with myriocin, an inhibitor of serine palmitoyl tranferase 1 which decreases skeletal muscle CER content [54]. However, the role of CER in insulin resistance appears restricted to saturated fat, as myriocin prevents skeletal muscle insulin resistance after infusion of palmitate but not oleate [55].

CER seems to activate atypical PKC isoforms such as PKCξ, and the protein phosphatase 2A (PP2A), leading, respectively, to the phosphorylation of Akt on a Thr34 residue decreasing its ability to bind to membrane PI3P and to the dephosphorylation of Ser473 activating residue, thus impairing insulin-induced activation of Akt, and subsequent translocation of GLUT4 vesicles to the plasma membrane [56, 57].

Molecular mechanisms leading to intramyocellular triglycerides accumulation

Defects in muscle fatty acids storage

IMTG emerge from circulating fatty acids, and their uptake inside muscle fibers is partly regulated by cluster of differenciation 36 (cd36) and fatty acid transport protein 1 (FATP1) transporters. Knock-out studies in mice have shown that CD36 [58, 59] and FATP1 [60] deletion protects mice from muscle lipid accumulation and muscle insulin resistance under high-fat diet. The first step of TAG synthesis is controlled by glycerol-3-phosphate acyltransferase (GPAT), which catalyzes the formation of lysophosphatidic acid from fatty acyl CoA and glycerol-3-phosphate. The final step of TAG synthesis is catalyzed by diacylglycerol

acyltransferases 1 and 2 (DGAT1 and DGAT2), which acylate DAG into TAG. Muscle-specific DGAT1 [61] or DGAT2 [62] overexpression leads to an accumulation of TAG in skeletal muscle, concomitant with a decrease in DAG content. In addition, unilateral overexpression of DGAT1 in rat skeletal muscle increased TAG content and rescued muscle insulin sensitivity when animals were fed high-fat diet [63]. Recently, Sparks and coworkers showed that muscle homogenates and human primary myotubes from obese individuals with T2D exhibit a reduced ability to incorporate FA into TAG pools [64]. Altogether, these studies indicate that increased rates of esterification of FA into TAG pools could be beneficial for insulin sensitivity by lowering lipotoxic lipid pressure in skeletal muscle.

Dysregulation of lipid droplets homeostasis

Perilipins

LDs are dynamic organelles, composed of a neutral lipid core surrounded by a phospholipid monolayer [65–67]. The surface of LDs is coated by different membrane-associated proteins, and the major structural components of LDs belong to the family of perilipins. Five members of the perilipin family have been identified so far, with various tissue expression patterns. In skeletal muscle, the most represented isoforms are PLIN2, PLIN3, PLIN4 and PLIN5 [68]. By forming a "lipolytic barrier", they also protect LDs against their hydrolysis by lipases. PLIN5 is highly expressed in oxidative tissues such as skeletal muscle, and PLIN5 knock-out mice display an accumulation of CER in muscle fibers and skeletal muscle insulin-resistance [69]. However, upon specific conditions, perilipins could also facilitate lipolysis. For instance, in adipose tissue, PLIN1 facilitates lipases access to LDs upon adrenergic stimulation of lipolysis [70–72]. In skeletal muscle, PLIN5 may play a role in fatty acids channeling to mitochondria, promoting by this way their oxidation upon stimulated conditions [73]. Indeed, independent studies from two different groups have demonstrated that PLIN5 also localizes to mitochondria, providing a physical linkage between LDs and mitochrondria [74, 75]. Further work is needed to clarify the physiological role of perilipins in skeletal muscle, with emphasis on the apparent discrepancy between resting and stimulated conditions.

Lipases

IMTG breakdown (i.e. lipolysis) is controlled by different enzymes, and their activity is finely regulated. The lipolytic machinery is highly expressed in red oxidative compared to white glycolytic muscle (Figure 2), and this is correlated with both IMTG content and oxidative capacity (unpublished data). The first step of IMTG breakdown is catalyzed by adipose triglyceride lipase (ATGL), which converts TAG into DAG. Then, hormone-sensitive lipase (HSL) converts DAG into MAG, and MAG are finally hydrolyzed by the monoacylglycerol lipase (MGL) [76]. At the end, lipolysis of one TAG molecule releases three fatty acid molecules and one glycerol molecule. Although HSL and MGL were the first lipases identified in skeletal muscle, and are both highly expressed, ATGL appears to be the first and rate limiting step of skeletal muscle lipolysis.

Monoacylglycerol lipase

MGL was discovered in 1976 by Tornqvist and colleagues, isolated from rat adipose tissue, and shown to specifically hydrolyze MAG but not TAG and DAG [77, 78]. MGL-knockout mice exhibit an increased MAG content associated with a down-regulation of free fatty acid and glycerol release from white adipose tissue [79]. MGL deletion also protects from diet-induced insulin-resistance and its inhibition in diabetic mice improves glucose tolerance [79, 80]. However, the physiological role of MGL in skeletal muscle lipolysis has not been investigated so far.

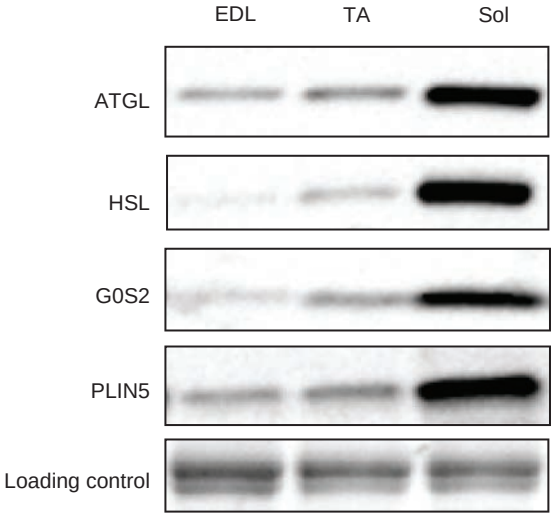

Figure 2: Lipolytic proteins are highly expressed in oxidative compared to glycolytic muscles.
Representative blot of protein expression of the major lipolytic proteins in glycolytic (EDL), mixed (TA) and oxidative (Sol) skeletal muscles. EDL, Extensor digitorum longus; TA, tibialis anterior; Sol, soleus; ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase; G0S2, G0/G1 switch gene 2; PLIN5, perilipin 5.

Hormone-sensitive lipase and adipose triglyceride lipase

Recent studies from our group have shown that diabetic status is associated with changes of ATGL and HSL activity and expression level in humans [81]. An increase of ATGL and a decrease of HSL expression/activity in skeletal muscle of obese and T2D patients was observed when compared to lean healthy individuals [82]. ATGL activity is negatively associated with whole-body insulin sensitivity measured by a hyperinsulinemic-euglycemic clamp [81]. Furthermore, ATGL protein expression has been shown to be increased in obese and T2D patients [81]. On the other hand, HSL expression and activity is down-regulated in obese insulin resistant subjects [82]. Of interest, this could be a primary defect acquired in obesity that persists in cultured human primary myotubes from obese type 2 diabetic individuals [83].

HSL was first identified in 1964 as a lipase sensitive to catecholamines in adipose tissue [84]. HSL presents TAG and DAG hydrolase activities, but displays a 10-times higher affinity for DAG rather than for TAG [76]. Confocal imaging studies have shown that HSL translocates to LDs in skeletal muscle upon stimulation by epinephrine or in response to contraction [85]. HSL-knockout mice accumulate DAG, but not TAG, in white adipose tissue and skeletal muscle, strengthening its role as a DAG hydrolase in vivo [86]. The observation that HSL-knockout mice do not accumulate TAG in skeletal muscle led to the conclusion that another enzyme is responsible for TAG-specific hydrolysis in skeletal muscle.

ATGL is highly expressed in skeletal muscle and white adipose tissue [87, 88], and ATGL mutations in humans lead to neutral lipid storage disease with myopathy [89]. Genetic modulations of ATGL expression in rodents helped to unravel its physiological role in vivo. Importantly, ATGL knockout mice display a massive TAG accumulation in cardiac and skeletal muscles [88]. In contrast, ATGL overexpression in human primary myotubes strongly decreases TAG content, while FA release and oxidation are increased [81]. More recent studies confirmed the important role of ATGL in skeletal muscle through muscle-specific modulations of its expression in mice [90, 91]. Collectively, these findings strengthen the role of ATGL as a TAG-specific hydrolase in skeletal muscle.

Mouse and cell models were helpful to define a causal relationship to strengthen the correlative findings linking ATGL to insulin resistance in human studies. Thus, ATGL knockout mice display a strong accumulation of TAG in skeletal muscle [88], as stated above, and are protected against high-fat diet induced insulin-resistance [92]. ATGL overexpression in human primary myotubes blunted insulin signalling and action. This phenotype was rescued when HSL was simultaneously overexpressed with ATGL, thus highlighting an important role for the balance between ATGL and HSL activity to maintain insulin sensitivity [81].

Enzymatic co-factors

ATGL activity is regulated by an enzymatic coactivator called comparative gene identification 58 (CGI58), which has also been shown to enhance TAG hydrolase activity in skeletal muscle [39]. CGI58 mutations in humans lead to the development of the Chanarin-Dorfman syndrome, associated with a severe myopathy [93]. CGI58 overexpression in human primary myotubes reduces TAG content and induces FA release and oxidation, whereas its down-regulation leads to TAG accumulation and reduces lipolysis [39]. It was demonstrated more recently that ATGL activity is also inhibited by G_0/G_1 switch gene 2 (G0S2) in adipocytes [94, 95]. Unpublished data from our group suggest that G0S2 also inhibits ATGL activity in human and mouse skeletal muscle, and further studies are necessary to better characterize its functional role.

Concluding remarks

Even if skeletal muscle lipids are undoubtedly linked to insulin resistance, which lipid species are responsible for the impairment of insulin signaling and action is still a matter of ongoing debate. Unlike what was originally proposed, IMTG per se do not seem to induce insulin resistance, and IMTG content is a predictor for T2D development only in sedentary individuals. In highly trained individuals, IMTG content is increased to match the energy demand of the exercising muscle, thus disconnecting IMTG from insulin resistance. In this case, muscle oxidative capacity seems to be a better predictor of insulin resistance than lipid content. This is explained by the fact that a mismatch between skeletal muscle lipolysis and oxidative capacity leads to the accumulation of lipotoxic species, such as DAG and CER, impairing insulin action. Of importance, other molecular mechanisms besides lipotoxicity might contribute to insulin resistance in metabolic diseases as reviewed extensively [4]. For instance, saturated fat and/or endotoxin-mediated inflammation could also trigger insulin resistance in liver and skeletal muscle [96, 97]. Endoplasmic reticulum and oxidative stress may also play a role in some organs while their direct role in mediating insulin resistance in skeletal muscle is still debated [98]. Although no direct evidence of apoptosis are visible in mouse skeletal muscle in response to lipid infusion and

chronic high fat feeding [4], ceramides can induce apoptosis by activating caspase 3 in a number of cell type such as beta cells [99]. Thus all these mechanisms are not mutually exclusive in mediating skeletal muscle insulin resistance and could be interconnected. Although it is now widely accepted that DAG and CER are key mediators of insulin resistance in skeletal muscle, the molecular mechanisms by which these lipotoxic lipids emerge in skeletal muscle remain unclear and need further investigation.

Expert opinion

During the last two decades the field has evolved with the concept that ectopic lipid storage in skeletal muscle can cause insulin resistance. It is now clear from a number of studies that not all lipids are alike and have the potential to inhibit insulin signaling and action. Thus non esterified fatty acids incoming to the muscle are neutralized within LD as IMTG. One current view, supported by in vitro data in human primary myotubes and in vivo work in transgenic mice, proposes that disturbances in LD dynamics could facilitates the emergence of toxic lipids, i.e. lipotoxicity, and insulin resistance. This includes alterations in lipases expression/activity and in LD proteins such as perilipins like PLIN2 and PLIN5. Because studies describing the link between muscle PLIN expression and insulin resistance are mostly correlative, future studies should examine the underlying molecular mechanisms in vitro as well as in vivo in mice with muscle-specific manipulations of PLIN in particular, and LD proteins in general. This area of research may help identify novel players in insulin resistance and design drug therapies to combat this strong risk factor of T2D in obese individuals.

Outlook

- LD is now recognized as a complex organelle with a specific proteome. Understanding if and how the LD proteome influences its dynamic and potential FA flux in and out the LD is a key challenge for the next decade. It is predictable that novel specific LD proteins will be identified in skeletal muscle and may behaves as potential druggable targets to combat insulin resistance.
- Although a number of clinical studies now underscore the potential importance of IMAT in metabolic diseases and aging, little is known on this ectopic fat depot and the biology of intramuscular adipocytes. It will be important in the near furture to investigate if IMAT is causally related to muscle dysfunction and how this depot emerges in the context of metabolic diseases and aging.

Highlights

- Intramuscular lipids are stored either as adipocytes between muscle fibers or as LD within muscle fibers
- Skeletal muscle lipid content is a stronger predictor of insulin resistance than circulating fatty acids in sedentary individuals
- The pathophysiological role of adipocytes located in skeletal muscle remains to be elucidated
- DAG and CER impair insulin signaling by activating PKC and PP2A
- Elevated ATGL activity in skeletal muscle contributes to lipotoxicity and insulin resistance
- HSL expression is reduced in skeletal muscle and primary myotubes from obese and T2D subjects
- Disturbances in LD dynamics and/or LD protein expression influences lipotoxicity and insulin sensitivity
- PLIN5 protein expression in skeletal muscle is a determinant of insulin sensitivity

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PUBLICATION 5 : L'ENTRAINEMENT EN ENDURANCE AUGMENTE L'EXPRESSION DES PROTEINES LIPOLYTIQUES ET REDUIT LA QUANTITE DE TRIACYLGLYCEROLS DANS LE MUSCLE SQUELETTIQUE DE SUJETS OBESES

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Endurance Exercise Training Up-Regulates Lipolytic Proteins and Reduces Triglyceride Content in Skeletal Muscle of Obese Subjects

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Context: Skeletal muscle lipase and intramyocellular triglyceride (IMTG) play a role in obesity-related metabolic disorders.

Objectives: The aim of the present study was to investigate the impact of 8 weeks of endurance exercise training on IMTG content and lipolytic proteins in obese male subjects.

Design and Volunteers: Ten obese subjects completed an 8-week supervised endurance exercise training intervention in which vastus lateralis muscle biopsy samples were collected before and after training.

Main Outcome Measures: Clinical characteristics and ex vivo substrate oxidation rates were measured pre- and posttraining. Skeletal muscle lipid content and lipolytic protein expression were also investigated.

Results: Our data show that exercise training reduced IMTG content by 42% ($P < .01$) and increased skeletal muscle oxidative capacity, whereas no change in total diacylglycerol content and glucose oxidation was found. Exercise training up-regulated adipose triglyceride lipase, perilipin (PLIN) 3 protein, and PLIN5 protein contents in skeletal muscle despite no change in mRNA levels. Training also increased hormone sensitive–lipase Ser660 phosphorylation. No significant changes in comparative gene identification 58, G_0/G_1 switch gene 2, and PLIN2 protein and mRNA levels were observed in response to training. Interestingly, we noted a strong relationship between skeletal muscle comparative gene identification 58 and mitochondrial respiratory chain complex I protein contents at baseline ($r = 0.87$, $P < .0001$).

Conclusions: Endurance exercise training coordinately up-regulates fat oxidative capacity and lipolytic protein expression in skeletal muscle of obese subjects. This physiological adaptation probably favors fat oxidation and may alleviate the lipotoxic lipid pressure in skeletal muscle. Enhancement of IMTG turnover may be required for the beneficial metabolic effects of exercise in obesity. **(*J Clin Endocrinol Metab* 98: 4863–4871, 2013)**

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Abbreviations: AMPK, 5′-AMP protein kinase; ATGL, adipose triglyceride lipase; CGI-58; comparative gene identification 58; DAG, diacylglycerol; HSL, hormone-sensitive lipase; IMTG, intramyocellular triglyceride; LD, lipid droplet; PLIN, perilipin; TAG, triacylglycerol; T2DM, type 2 diabetes mellitus; V_{O_2}max, maximal oxygen uptake.

Obesity and type 2 diabetes mellitus (T2DM) have been linked to ectopic fat deposition in nonadipose tissues such as skeletal muscle, which plays an important role in the etiology of insulin resistance (1). Ectopic lipids mainly accumulate as triacylglycerols (TAGs) stored within lipid droplets (LDs) in skeletal muscle. Intramyocellular triglyceride (IMTG) pools are now recognized as an important fuel source during exercise (2). IMTG content is increased in athletes and in lean healthy subjects in response to exercise training interventions (3, 4). However, IMTG content is paradoxically elevated in obese and T2DM individuals (5), and an inverse association between IMTG content and peripheral glucose disposal has been repeatedly reported in sedentary subjects (5, 6). It is now well established that IMTG is associated with elevated levels of lipotoxic intermediates such as diacylglycerols (DAGs) and ceramides disrupting insulin-stimulated glucose disposal (1). However, it is not clear so far how IMTG mediates lipotoxicity in obese sedentary subjects. LDs are dynamic organelles resulting from the balance between storage and breakdown of TAGs by lipases. We have recently shown that disturbances in lipase expression/activity in skeletal muscle may contribute to lipotoxicity and insulin resistance (7). Adipose triglyceride lipase (ATGL) expression is up-regulated, whereas hormone-sensitive lipase (HSL) expression is reduced in the muscle of obese subjects (8). A causal relationship between elevated ATGL and/or reduced HSL activity and insulin resistance was shown in vitro in human primary myotubes (7). ATGL is the main TAG hydrolase in mouse and human skeletal muscle (7, 9), whereas HSL exhibits a strong preference for DAG (10). ATGL activity is acutely regulated by comparative gene identification 58 (CGI-58) in human skeletal muscle (11). Recent data indicate that ATGL activity is inhibited by G_0/G_1 switch gene 2 (*G0S2*) (12), but nothing is known about its expression and potential role in human muscle. Both ATGL and HSL activity could also be regulated by LD-associated proteins of the perilipin (PLIN) family. At least 3 main isoforms are expressed in human skeletal muscle (PLIN2/3/5). Recent studies in transgenic mice with whole ablation or cardiac-specific modulations of PLIN5 have been insightful in determining the metabolic role of PLIN5 in skeletal and cardiac muscles (13, 14). PLIN5 seems to protect LDs from TAG hydrolysis and may channel fatty acids to mitochondrial β-oxidation. PLIN2 has also been associated with IMTG and insulin sensitivity in humans (15). Although PLIN3 is expressed in human muscle fibers (16), little is known so far about its function in skeletal muscle. Taken together, these results suggest that lipolytic proteins may play a role in the regulation of oxidative metabolism, lipotoxicity, and insulin sensitivity. Lifestyle interventions incorporating increased physical activity remain the primary preventive approach for metabolic diseases such as obesity and T2DM (17). Exercise training improves whole-body insulin sensitivity and metabolic flexibility in obese individuals and individuals with T2DM (18). One possible mechanism by which exercise training improves insulin sensitivity is by restoring lipase expression and reducing lipid content in skeletal muscle. Thus, enhancement of IMTG turnover may be required for the beneficial metabolic effects of exercise in obesity. The purpose of this study was to investigate the effect of 8 weeks of endurance exercise training on skeletal muscle neutral lipid content and lipolytic proteins in middle-aged obese male individuals.

Materials and Methods

Subjects

Nineteen male obese subjects (mean age 36 ± 2 years; mean body mass index, 32.3 ± 0.7 kg/m^2; range, 29.4–35.8 kg/m^2) were recruited at the Toulouse Clinical Investigation Centre. Subjects were included if they had a body mass index <40 kg/m^2, blood pressure <140/90 mm Hg, and normal thyroid function. Participants were excluded if they had history of diabetes, cardiovascular diseases, or cancer or if they had any contraindication for local anesthetics and exercise. They were weight stable for the last 3 months and free of medications, chronic diseases, and known comorbidities. Ten subjects participated in an 8-week supervised endurance exercise training program in which paired biopsies were performed at rest before and after training. The participants were asked to refrain from vigorous physical activity for 48 hours before presenting to the clinical investigation center and ate a weight-maintaining diet consisting of 35% fat, 16% protein, and 49% carbohydrates 2 days before the experiment. Dietary intake was assessed by a dietician from a 3-day weighed food record, including 2 weekdays and 1 weekend day, the week before the first investigation day. Dietary records were assessed at baseline and during the last week of the program. Nutrient intake was calculated using PRoFIL software (version 6.7; Audit Conseil en Informatique Médicale) with the CIQUAL French food composition database for diet composition. Self-reported food intake did not change significantly during the training program (2567 ± 211 vs 2228 ± 158 kcal/d for pre- and posttraining, respectively).

Ethics statement

This study was performed according to the latest version of the Declaration of Helsinki and the current International Conference on Harmonization guidelines. The application was approved by the Toulouse University Hospitals Ethics Committee, and all subjects gave written informed consent. The research was conducted at the Clinical Investigation Centre of Toulouse Hospitals under the supervision of trained staff and used standardized procedures.

Study design

Body composition, blood parameters, and maximal oxygen uptake (Vo_2max) were measured on one experimental day, and muscle biopsies and oral glucose tolerance tests (OGTT) were performed on a second experimental day 1 week apart. On each experimental day, subjects were investigated after a 10-hour overnight fast. Vo_2max was assessed using a graded exercise test conducted on an electromagnetically braked bicycle ergometer (Ergometrics 800; Ergoline) as described previously (19). The initial workload was 50 W, and it was increased by 30 W every 3 minutes until exhaustion. Heart rate was continuously monitored by telemetry using a heart rate monitor (Ergocard). We considered that the subjects achieved their Vo_2max when all of the following usual and accepted criteria were achieved: maximal heart rate measured at exhaustion was higher than 90% of the age-predicted maximal heart rate, respiratory quotient (ie, Vco_2/Vo_2) measured at exhaustion was higher than 1.1, and the subjects could not sustain a sufficient rate of cycling. Breath-by-breath measurements were taken at rest and throughout exercise to assess air flow and O_2 and CO_2 concentrations in expired gases by using a computerized ergospirometer (Ultima PFX; Medical Graphics). The Vo_2max exercise trial was performed in a ventilated room to ensure a constant room temperature and hygrometry from the calibration just before the trial. Muscle biopsy samples from vastus lateralis weighing 60 to 100 mg each were obtained using the Bergstrom technique (20). Pieces of muscle were collected in the respective buffers for ex vivo oxidation and glucose transport assays or blotted, cleaned, and snap-frozen in liquid nitrogen for gene expression and Western blot analyses. Ninety minutes after biopsy sampling, a 75-g oral glucose tolerance test (according to World Health Organization standards) was performed, and blood samples were collected at 0, 15, 30, 45, 60, 90, and 120 minutes. Body composition (considering a 3-compartment model) was determined before and after training using a total-body dual-energy x-ray absorptiometer (DPX software 3.6; Lunar Radiation Corp). Blood glucose was assayed using the glucose oxidase technique (bioMérieux), and plasma insulin was measured using a Bi-insulin IRMA kit (Bertin Pharma). Plasma free fatty acids were assayed with an enzymatic method (Wako kit; Unipath). Plasma cholesterol and triglycerides were determined using standard clinical biochemistry methods.

Exercise training

Endurance exercise was performed at the Centre de Ressources, d'Expertise et de Performance Sportives (CREPS) of Toulouse (Toulouse, France). Exercise sessions consisted mainly of cycling and running, 5 times per week for 8 weeks. Subjects exercised 3 times per week under supervision by an experienced physical coach during the first 4 weeks and 2 times per week during the last 4 weeks. They exercised on their own during other sessions. All daily sessions consisted of at least a 20-minute warm-up at 35% Vo_2max followed by progressively increasing exercise intensity (up to 85% Vo_2max) and duration (up to 1 hour) throughout the training program. The subjects exercised at a target heart rate corresponding to 35% to 85% of their Vo_2max. Heart rate was monitored with a Suunto T3 Cardiometer (MSE). Compliance with training was good, as checked by a training diary including day-to-day activities. The percentage of sessions completed was >85%.

Palmitate and glucose oxidation in muscle homogenates

This assay was performed as described previously (21, 22). In brief, 50 mg of muscle was minced and homogenized in a modified sucrose-EDTA medium (250 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl [pH 7.4]). Palmitate oxidation rates were determined by measuring production of ^{14}C-labeled acid-soluble metabolites (ASMs), a measure of tricarboxylic acid cycle intermediates and acetyl esters (incomplete oxidation), and $[^{14}C]CO_2$ (complete oxidation). The glucose oxidation rate was determined by measuring $[^{14}C]CO_2$. The radioactivities of CO_2 and ASMs were determined by liquid scintillation counting. Data are expressed as nanomoles per hour and were normalized per gram of tissue weight.

Determination of neutral lipid content

Muscle tissue was homogenized in 1 mL of methanol-5 mM EGTA (2:1, v/v) with Fast-Prep (MP Biochemicals). Lipids corresponding to 20 mg of tissue were extracted according to Bligh and Dyer (23) in dichloromethane-methanol-water (2.5:2.5:2.1, v/v/v), in the presence of the internal standards (3 μg of stigmasterol, 3 μg of 1,3-dimyristine, 3 μg of cholesteryl heptadecanoate, and 20 μg of glyceryl trinonadecanoate) as described previously (21). The equivalent of 0.3 mg of tissue was evaporated under nitrogen, the dry pellets were dissolved overnight in 0.2 mL of NaOH (0.1 M), and proteins were measured with the Bio-Rad protein assay.

Reverse transcription and real-time quantitative PCR

Total RNA from muscle tissue was isolated with an RNeasy mini kit according to the manufacturer's instructions (QIAGEN GmbH). The quantity of RNA was determined on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). RT-PCR was performed on a GeneAmp PCR System 9700 using 1 μg

Table 1. Clinical and Biochemical Variables in Obese Subjects at Baseline (Pretraining) and After 8 Weeks (Posttraining) of Endurance Exercise Training

	Pretraining	Posttraining	*P* Value
Body weight, kg	102.1 ± 2.2	102.3 ± 2.4	NS
Body mass index, kg/m^2	32.3 ± 0.7	32.4 ± 0.7	NS
Fat mass, %	34.5 ± 1.6	33.7 ± 1.8	.083
Lean body mass, kg	62.9 ± 1.9	63.9 ± 2.4	.087
Vo_2max, L/min	2.8 ± 0.1	3.1 ± 0.2	.007
Vo_2max, mL/min/kg	26.7 ± 0.7	28.7 ± 1.3	.023
Fasting insulin, μU/mL	16.5 ± 2.3	13.5 ± 1.9	NS
Fasting glucose, g/L	0.90 ± 0.03	0.90 ± 0.02	NS
AUC_{0-120} glucose	168 ± 6	167 ± 7	NS
AUC_{0-120} insulin	9150 ± 1543	10846 ± 1965	NS
Plasma cholesterol, mM	5.18 ± 0.44	5.05 ± 0.41	NS
Plasma triglycerides, mM	1.34 ± 0.13	1.21 ± 0.17	NS
Plasma free fatty acids, μM	381 ± 38	396 ± 42	NS

Abbreviation: AUC, area under the curve; NS, not significant. Values are means ± SEM (n = 10).

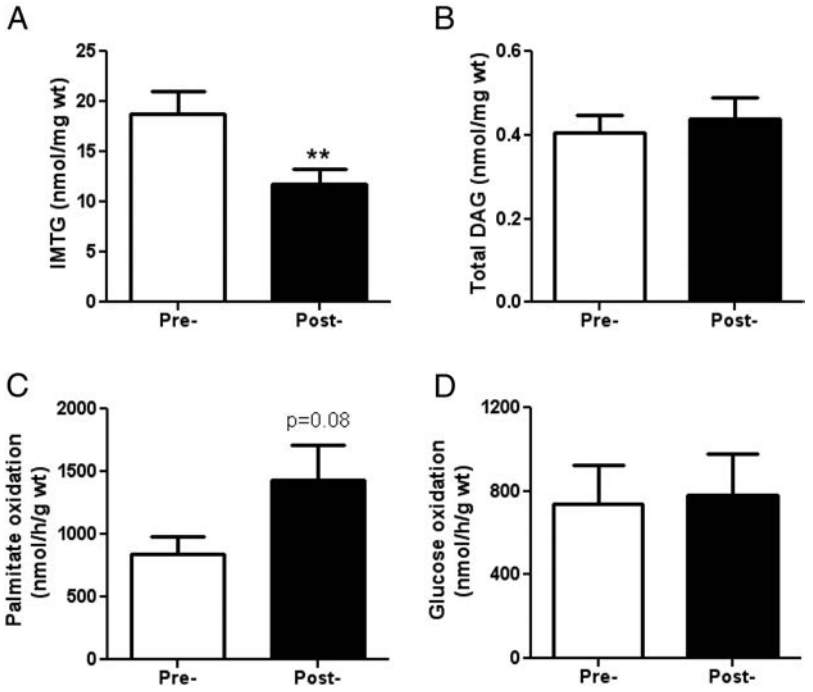

Figure 1. Effect of exercise training on skeletal muscle lipid content and substrate oxidation. IMTG (A) and total DAG (B) contents were measured in vastus lateralis biopsy samples before (Pre-) and after (Post-) exercise training. Palmitate oxidation (C) and glucose oxidation (D) were measured ex vivo on fresh muscle tissue from obese subjects before and after exercise training. **, $P < .01$, comparing the effect of exercise training.

of RNA and the MultiScribe reverse transcriptase method (Applied Biosystems). Real-time quantitative PCR was performed to determine cDNA content. Primers from Applied Biosystems were as follows: *18S* (TaqMan assay identification number: Hs99999901_s1), *PNPLA2* (patatin-like phospholipase domain-containing protein 2, also called ATGL, Hs00982040_g1), *LIPE* (hormone-sensitive lipase, Hs00943404_m1), *CGI-58* (comparative gene identification 58, Hs00211205_m1), *G0S2* (G_0/G_1 switch gene 2, Hs00274783_s1), *PLIN2* (perilipin 2, Hs00605340_m1), *PLIN3* (perilipin 3, HS00998416_m1), and *PLIN5* (perilipin 5, Hs00965990_m1). The amplification reaction was performed in duplicate on 10 ng of the cDNA samples in a final volume of 20 μL in 96-well reaction plates on a StepOnePlus real-time PCR system (Applied Biosystems). All reactions were performed under the same conditions: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. All expression data were normalized by the $2^{\Delta C_t}$ method using 18S rRNA as an internal control.

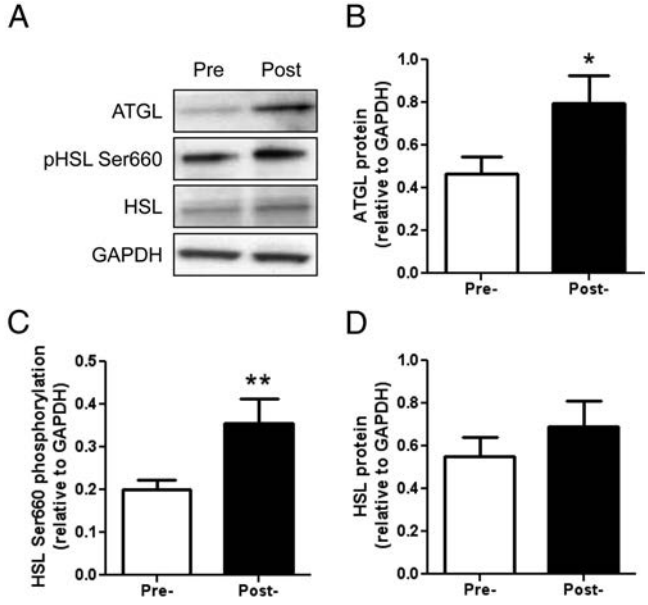

Figure 2. Effect of exercise training on skeletal muscle lipases. A, Representative blots of ATGL, HSL, HSL Ser660, and GAPDH phosphorylation. B–D, Quantitative bar graphs of ATGL protein (B), HSL Ser660 phosphorylation (C), and HSL protein (D) in skeletal muscle of obese subjects before (Pre-) and after (Post-) exercise training. *, $P < .05$; **, $P < .01$, comparing the effect of exercise training.

Western blotting

Muscle tissue pieces were homogenized in a buffer containing 50 mM HEPES (pH 7.4), 2 mM EDTA, 150 mM NaCl, 30 mM $NaPO_4$, 10 mM NaF, 1% Triton X-100, 10 μL/mL protease inhibitor (Sigma-Aldrich), 10 μL/mL phosphatase I inhibitor (Sigma-Aldrich), 10 μL/mL phosphatase II inhibitor (Sigma-Aldrich), and 1.5 mg/mL benzamidine HCl. Tissue homogenates were centrifuged for 25 minutes at 15 000 $\times$ *g*, and supernatants were stored at −80°C. Solubilized proteins (40 μg) were run on 4% to 12% SDS-PAGE (Bio-Rad), transferred onto a nitrocellulose membrane (Hybond ECL; Amersham Biosciences), and incubated with the primary antibodies: ATGL, HSL, pHSL Ser660, Akt, IRS1, AMPK (Cell Signaling Technology), OXPHOS (MitoSciences), CGI58 (Abnova Corp), and G0S2 (ProteinTech). For detection of PLINs, membranes were probed with PLIN2 and PLIN3 (Thermo Scientific), and PLIN5 (Progen) antibodies. Subsequently, immunoreactive proteins were visualized using a ChemiDoc MP Imaging System (Bio-Rad), and data were analyzed using Image Lab software (version 4.1; Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (Cell Signaling Technology) served as an internal control.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 5.0 for Windows (GraphPad Software Inc). Normal distribution and homogeneity of variance of the data were tested using the Shapiro-Wilk and F tests, respectively. Paired Student *t* tests were performed to determine the effect of training on anthropometric and clinical variables (Table 1). Pearson correlations were run to assess relationships between lipases and PLIN mRNA and proteins. Univariate linear regressions were applied to evaluate the relationship between ex vivo palmitate oxidation, complex I protein, and CGI-58 protein. All values in figures and the table are presented as means ± SEM. Statistical significance was set at a value of $P < .05$.

Results

Exercise training improves whole-body aerobic capacity and muscle glucose uptake

The 8-week training intervention tended to reduce body fat mass and to increase lean body mass, and it significantly increased Vo_2max by about 10% (Table 1). The exercise intervention also tended to reduce the fasting plasma insulin level (−18%, $P = .07$) with no change in fasting glucose (Table 1). This result was associated with higher Akt Ser473 phosphorylation (3.4-fold), Akt total protein content (1.75-fold), and basal glucose uptake (2.65 fold) in skeletal muscle (Supplemental Figure 1 published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org.). No change in 5′-AMP protein kinase (AMPK) total protein expression was observed in response to training (Supplemental Figure 1). The higher basal glucose uptake occurred in light of significantly elevated phosphorylation of the Akt substrate of 160 kDa (AS160) (3.3-fold), whereas no significant changes in skeletal muscle insulin receptor substrate-1 phosphorylation and total protein were observed (Supplemental Figure 2). No changes in the mRNA levels of the main PI3K subunits expressed in skeletal muscle were noted posttraining (data not shown). No significant effect of training on glucose tolerance and plasma lipids was observed (Table 1).

Exercise training increases muscle fat oxidative capacity and reduces lipid content

We next assessed the impact of the 8-week exercise training intervention on neutral lipid content in skeletal muscle. Exercise training strongly reduced resting IMTG content (−42%, $P = .008$) (Figure 1A), whereas no significant change in total DAG content was observed (Figure 1B). Interestingly, palmitate oxidation rates measured ex vivo in fresh muscle tissue tended to increase in response to training (+71%, $P = .08$) (Figure 1C), whereas glucose oxidation remained unchanged (Figure 1D). Mitochondrial respiratory chain complex I (0.50 ± 0.08 vs 0.37 ± 0.05 arbitrary units, $P < .05$), complex III (0.45 ± 0.07 vs 0.27 ± 0.04 arbitrary units, $P < .01$), and complex V (1.59 ± 0.17 vs 1.28 ± 0.15 arbitrary units, $P < .05$) protein contents in skeletal muscle were also significantly up-regulated posttraining.

Effect of endurance exercise training on skeletal muscle lipase expression

All biopsy samples analyzed were negative for PLIN1, excluding significant contamination by adipocytes (data not shown). Western blotting analyses on lysates of vastus lateralis muscle biopsy samples revealed a comparable increase in both ATGL protein (+72%, $P = .04$) (Figure 2, A and B) and HSL Ser660 phosphorylation (+78%, $P = .02$) (Figure 2, A and C). This occurred in the absence of a statistical change in total HSL protein (Figure 2, A and D). The higher HSL Ser660 phosphorylation observed after training remained significant after adjustment for total HSL protein (0.45 ± 0.08 vs 0.57 ± 0.09 arbitrary units, $P = .04$). Of note, no significant changes in ATGL (*PNPLA2*) and HSL (*LIPE*) gene expression were observed in response to training (Supplemental Table 1).

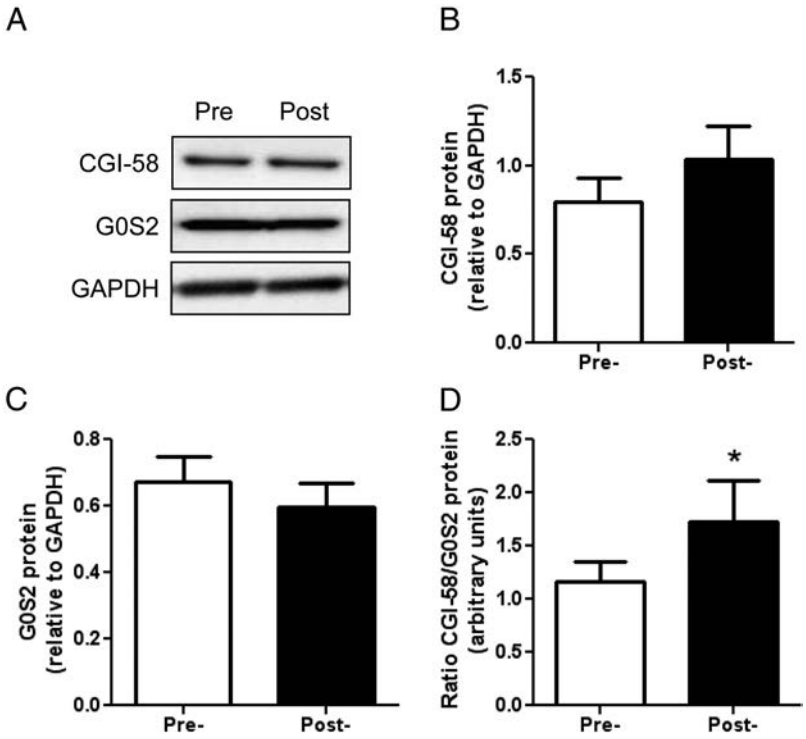

Figure 3. Effect of exercise training on skeletal muscle colipases. A, Representative blots of CGI-58, G0S2, and GAPDH. B–D, Quantitative bar graphs of CGI-58 protein (B), G0S2 protein (C) and the ratio of CGI-58 to G0S2 protein (D) in skeletal muscle of obese subjects before (Pre-) and after (Post-) exercise training. *, $P < .05$, comparing the effect of exercise training.

Effect of endurance exercise training on skeletal muscle colipase expression

Because ATGL activity is actively controlled by CGI-58 and G0S2, we next assessed the influence of exercise training on CGI-58 and G0S2 protein content in the skeletal muscle of obese subjects. We could show that exercise training did not significantly affect either CGI-58 (+20%, not significant) (Figure 3, A and B) or G0S2 (Figure 3, A and C) protein ex-

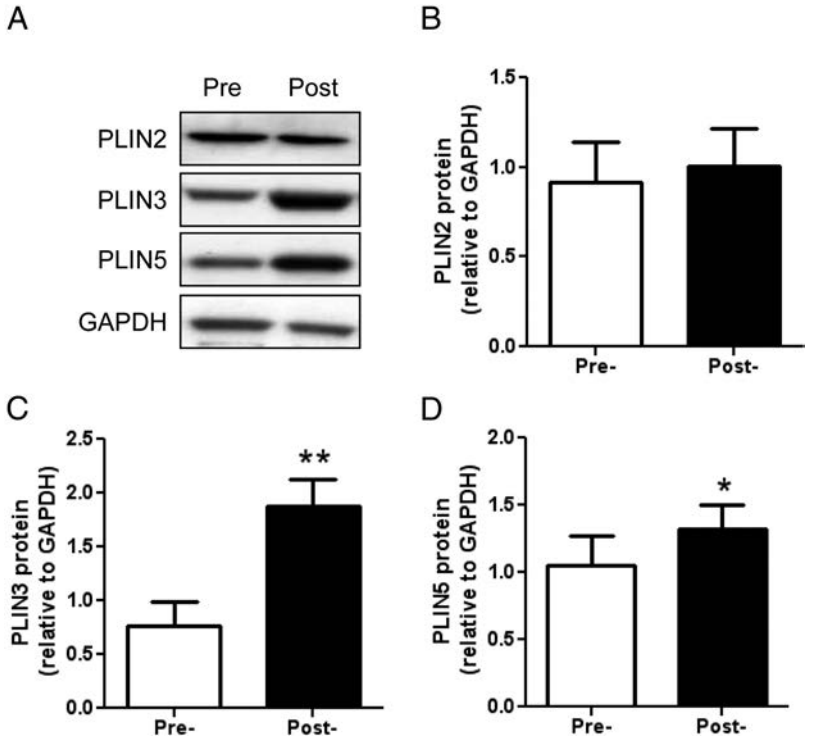

Figure 4. Effect of exercise training on skeletal muscle PLINS. A, Representative blots of PLIN2, PLIN3, PLIN5, and GAPDH phosphorylation. B–D, Quantitative bar graphs of PLIN2 protein (B), PLIN3 protein (C), and PLIN5 protein (D) in skeletal muscle of obese subjects before (Pre-) and after (Post-) exercise training. *, $P < .05$; **, $P < .01$, comparing the effect of exercise training.

pression. No changes in CGI-58 and G0S2 mRNA levels were observed (Supplemental Table 1). However, exercise training increased the ratio of CGI-58 to G0S2 protein (+48%, $P = .04$) (Figure 3D).

Effect of endurance exercise training on skeletal muscle PLIN expression

PLINs are LD-associated proteins playing a role in the regulation of lipolysis. We observed a significant up-regulation of skeletal muscle PLIN3 (2.4-fold, $P = .006$) (Figure 4, A and C) and PLIN5 (+26%, $P < .05$) (Figure 4, A and D) proteins in response to training. No significant effect on muscle PLIN2 protein content was noted (Figure 4, A and B). As reported for lipases and colipases, the training intervention did not affect PLIN2/3/5 gene expression (Supplemental Table 1). No correlation between PLIN, lipase and colipase protein, and mRNA levels was observed at baseline. However, a significant positive relationship between respiratory chain complex I protein and palmitate oxidation (Figure 5A) and a robust positive correlation between respiratory chain complex I protein and CGI-58 protein were found at baseline in our obese cohort (Figure 5B).

Discussion

Exercise training is known to improve insulin sensitivity and muscle fat oxidative capacity. The major and novel findings of the present study are (1) that exercise training coordinately up-regulates fat oxidative capacity and lipolytic proteins (ATGL, HSL Ser660, PLIN3, and PLIN5) which together reduce resting IMTG content in obese subjects and (2) that G0S2 is significantly expressed in human skeletal muscle and not regulated by exercise training. This physiological adaptation may be seen as a favorable metabolic event (1) to alleviate the lipotoxic lipid pressure in skeletal muscle during obesity and (2) to enhance IMTG breakdown and utilization as fuel during exercise.

We first observed that exercise training intervention was effective in increasing maximal oxygen consumption and also tended to improve whole-body insulin sensitivity as reflected by lower fasting plasma insulin concentration posttraining, in line with several studies (18, 24). Although we did not directly assess skeletal muscle insulin sensitivity, we observed a significant up-regulation of basal glucose uptake and of Akt and its downstream target AS160 activation involved in GLUT4 trafficking and glucose transport (25, 26). Although AMPK is known to promote glucose transport in skeletal muscle (27), the higher basal glucose transport after training appeared to be independent of substantial changes in skeletal muscle AMPK protein content. These data are in agreement with other studies reporting a significant effect of strength and/or interval training on total Akt but no effect on total AMPK protein in skeletal muscle (28, 29). The improvement in whole-body aerobic capacity was paralleled by an up-regulation of skeletal muscle oxidative capacity. We also show that palmitate oxidation rates were closely associated with complex I protein expression in skeletal muscle, thus reflecting a surrogate marker of maximal oxidative capacity in this tissue. These data are in line with recent studies reporting a net increase in muscle ex vivo palmitate oxidation rates in obese subjects (30) and in subjects with T2DM (22) in response to several weeks of exercise training and are also consistent with an up-regulation of mitochondrial biogenesis and respiratory chain complex protein expression in response to training (22, 26).

An increase in muscle fat oxidative capacity may contribute to improving metabolic health in obese subjects. A

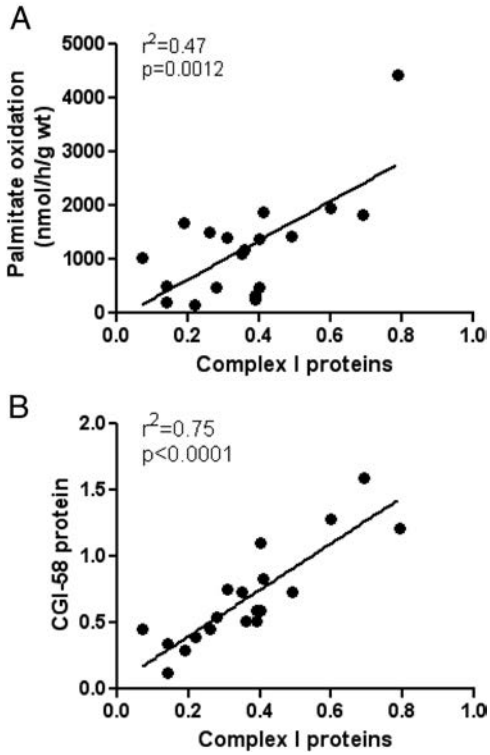

Figure 5. A, Correlation between skeletal muscle complex I protein expression and ex vivo palmitate oxidation rates at baseline (n = 19). B, Correlation between skeletal muscle complex I and CGI-58 protein expression at baseline (n = 19).

strong inverse relationship between muscle lipid content and whole-body insulin sensitivity has been widely documented in sedentary subjects (5, 6). We report here a strong reduction in IMTG resting content in muscle of obese subjects in response to endurance exercise. Overall, this finding is in agreement with other studies (31, 32). However, it is worth mentioning at this point that several training studies in lean healthy subjects have reported a net increase in total IMTG content (4, 24, 33). IMTG content is also elevated in endurance-trained individuals compared with that in age-matched controls (3) and is positively associated with insulin sensitivity in fit individuals (6). This observation is at the basis of the so called "athlete paradox" and the U-shaped relationship between IMTG content and insulin sensitivity (34). Thus, because IMTG content is increased several fold in obese and type 2 diabetic subjects (5, 6), the 42% decrease in total IMTG content observed in this study may not be sufficient to come back to the level observed in lean subjects. Of importance, the net decrease in IMTG may be due to a combination of increased fat oxidative capacity and lipase content. IMTG is a significant fuel source during exercise (2) and also potentially at rest because skeletal muscle largely relies on fat as a fuel during fasting (35). Consequently, IMTG lipolysis is a fine-tuning process to supply fatty acid fuels during periods of higher energy demand such as exercise.

Our data show a strong up-regulation of ATGL protein and HSL activation at Ser660 in skeletal muscle of obese subjects posttraining. This finding is in agreement with at least one other study in lean, healthy young men (31). HSL phosphorylation at Ser660 is mediated by cAMP-dependent protein kinase (36). HSL exhibits a strong preference for DAGs as substrates (10). However, the coordinated up-regulation of both ATGL and HSL did not significantly change muscle total DAG content in this study. This finding is in line with in vitro data showing that concomitant overexpression of ATGL and HSL does not significantly alter total DAG content (7). One study limitation here is that we did not directly measure membrane vs cytosolic DAG content, which may have been a better marker of bioactive DAG pools and insulin resistance (37). Muscle membrane DAG content is significantly reduced in athletes who are otherwise more insulin sensitive than matched control subjects. Thus, no significant changes in ATGL and HSL mRNA levels were observed in response to training. This result highlights the fact that lipases are actively regulated at the posttranscriptional level. ATGL is robustly activated by CGI-58 (38) and inhibited by G0S2 (12). We and others have shown that CGI-58 is highly expressed in human skeletal muscle and coactivates ATGL (11, 31). We also report for the first time significant expression of G0S2 at the mRNA and protein levels in human skeletal muscle. In this study, endurance training increased the ratio of CGI-58 to G0S2, which may result in an enhancement of ATGL activity.

Of note, we found a strong positive relationship between CGI-58 protein and mitochondrial respiratory chain complex I protein expression at baseline in our obese cohort. This finding suggests a tight coupling of lipolytic and mitochondrial fat oxidative capacity in skeletal muscle. We also observed a significant up-regulation of PLIN5, which is a known peroxisome proliferator–activated receptor-target gene, in muscle after training (39). PLIN5 is predominantly expressed in oxidative tissues such as cardiac and skeletal muscles and may actively control lipid storage and lipolysis in those tissues (13, 14). Our data are in agreement with another recent study showing increased muscle PLIN5 protein content in response to 6 weeks of endurance or sprint interval training in lean healthy volunteers (33). We also report a robust up-regulation of muscle PLIN3 and no change in PLIN2 protein expression after training. PLIN2 and PLIN3 are abundant PLIN proteins in human skeletal muscle (16, 33). PLIN2 is a constitutive LD-associated protein coating large LDs. On the other hand, PLIN3 is an exchangeable LD-associated protein that preferentially coats small LDs in response to lipolytic or lipogenic stimuli (40). Collectively, the up-regulation of PLIN proteins (PLIN3 and PLIN5)

may reduce access of the main lipases (ATGL and HSL) to LDs in the basal state (rest) and favor IMTG-derived fatty acid channeling to mitochondria in the stimulated state (exercise).

We acknowledge that our study may have been underpowered to capture significant changes in some secondary outcomes presented here. Potential contamination of muscle biopsy samples by infiltrated adipocytes was excluded by immunoblotting PLIN1, which is not present at the protein level in skeletal muscle (31). Skeletal muscle insulin sensitivity and signaling were not specifically assessed by a hyperinsulinemic-euglycemic clamp. The strength of the current study is the longitudinal intervention with detailed metabolic phenotyping of skeletal muscle ex vivo.

In summary, our study shows that aerobic exercise training enhances basal ATGL expression and HSL phosphorylation in human skeletal muscle. This physiological adaptation probably contributes to a reduction in resting IMTG content in obese subjects. Thus, the concomitant up-regulation of muscle fat oxidative capacity and lipolytic proteins in response to training, as well as the strong baseline relationship between these 2 parameters, highlights the fact that IMTG lipolysis is an important metabolic function of skeletal muscle. An enhancement in IMTG turnover may be required for the beneficial metabolic effects of exercise in obesity.

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Disclosure Summary: The authors have nothing to disclose.

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Supplemental Table 1. Skeletal muscle gene expression in obese subjects at baseline and after 8 weeks of aerobic exercise training.

Gene name	Pre-training	Post-training
PNPLA2 (ATGL)	14.3 ± 1.3	16.7 ± 1.3
LIPE (HSL)	0.7 ± 0.1	0.9 ± 0.2
CGI-58	1.9 ± 0.2	2.1 ± 0.1
G0S2	41.0 ± 8.4	42.8 ± 10.9
PLIN2	7.0 ± 1.0	6.8 ± 0.4
PLIN3	10.8 ± 1.2	11.2 ± 0.5
PLIN5	11.6 ± 2.5	11.8 ± 1.7

Values are given as mean ± SEM. Arbitrary units are given as $2^{\Delta Ct} \times 100000$ for all genes.

Figure 1. (A) Basal 2-deoxyglucose uptake in skeletal muscle pre- and post-exercise training. Quantitative bar graphs of (B) Akt Ser473 phosphorylation, (C) total Akt protein and (D) total AMPK protein in resting skeletal muscle of obese subjects pre- and post- of exercise training. Insets show representative blots of each protein. $^{\#}$ p=0.06, *** p<0.01 comparing the effect of exercise training.

Figure 2. Quantitative bar graphs of (A) IRS1 Tyr612 phosphorylation, (B) total IRS1 protein, (C) AS160 Thr642 phosphorylation relative to AS160 total protein, and (D) representative blots in resting skeletal muscle of obese subjects pre- and post- of exercise training. Insets show representative blots of each protein. * p<0.05, ** p<0.01 comparing the effect of exercise training.

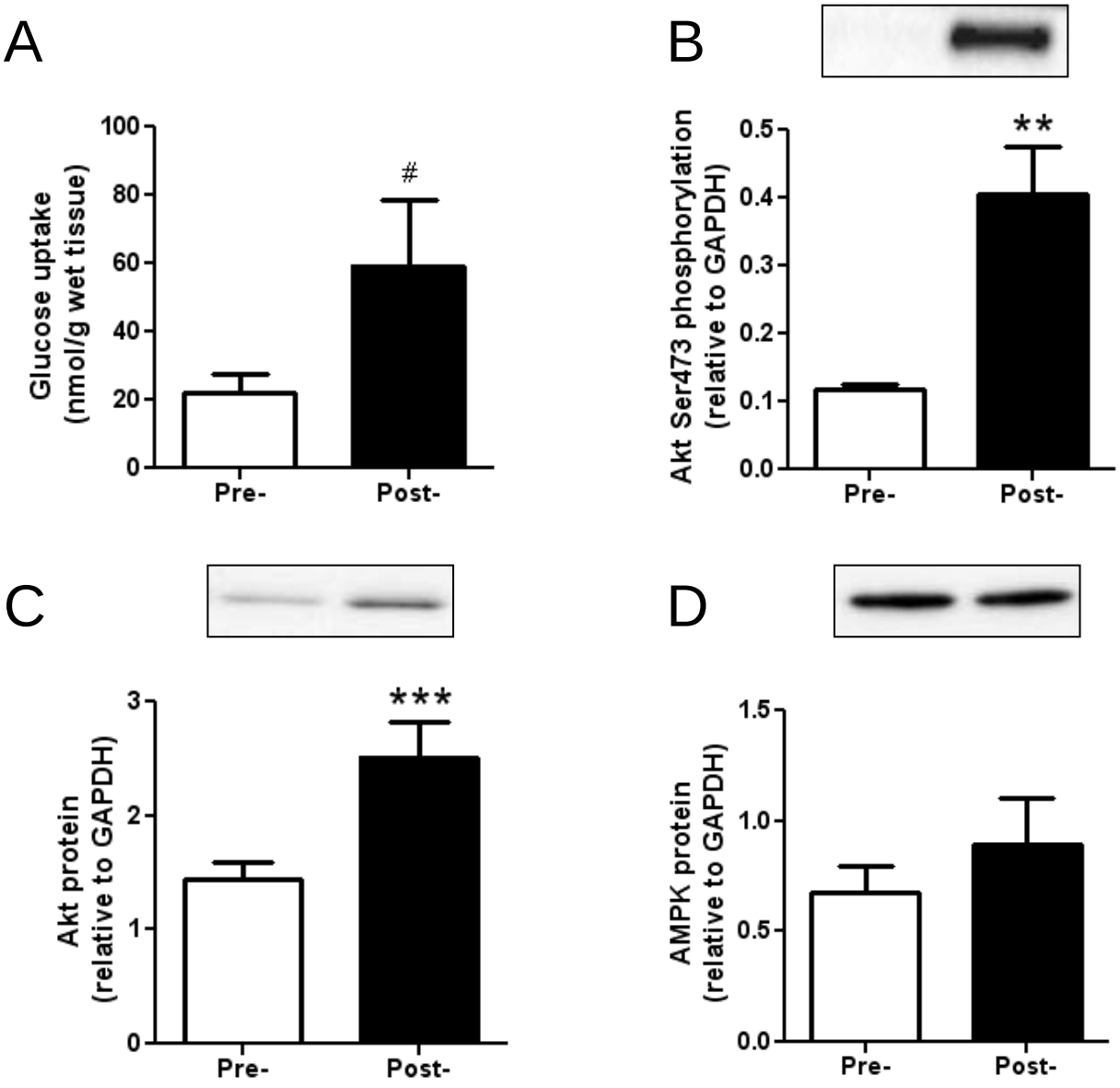
A
Glucose uptake (nmol/g wet tissue)
100
80
60
40
20
0
#
Pre-
Post-
B
Akt Ser473 phosphorylation (relative to GAPDH)
0.5
0.4
0.3
0.2
0.1
0.0
**
Pre-
Post-
C
Akt protein (relative to GAPDH)
3
2
1
0
***
Pre-
Post-
D
AMPK protein (relative to GAPDH)
1.5
1.0
0.5
0.0
Pre-
Post-

Figure 1

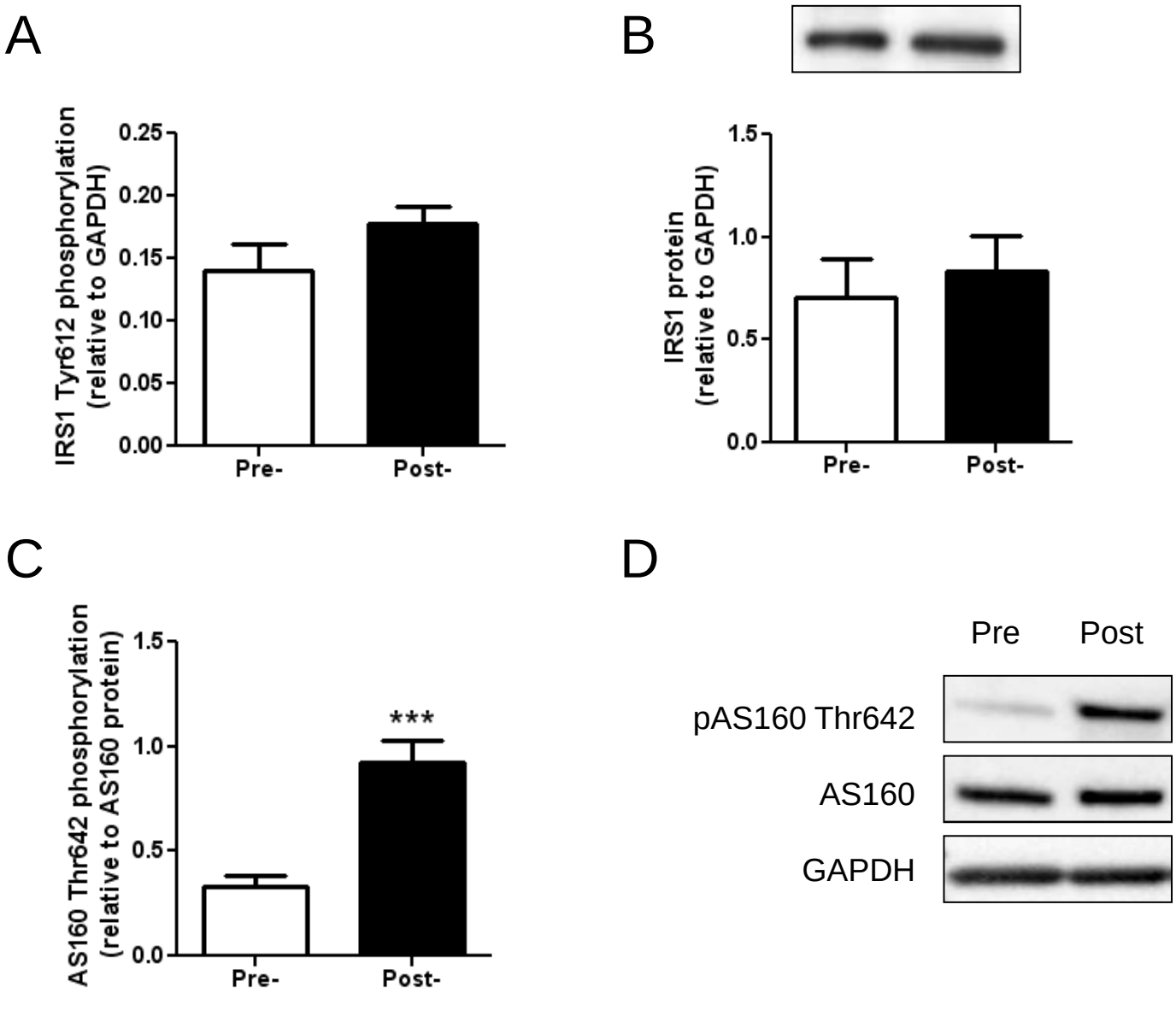
A
IRS1 Tyr612 phosphorylation (relative to GAPDH)
0.25
0.20
0.15
0.10
0.05
0.00
Pre-
Post-
B
IRS1 protein (relative to GAPDH)
1.5
1.0
0.5
0.0
Pre-
Post-
C
AS160 Thr642 phosphorylation (relative to AS160 protein)
1.5
1.0
0.5
0.0
***
Pre-
Post-
D
Pre
Post
pAS160 Thr642
AS160
GAPDH

Figure 2

PUBLICATION 6 : DEFAUTS PRIMAIRES DANS LA LIPOLYSE ET L'ACTION DE L'INSULINE DANS DES CELLULES MUSCULAIRES ISSUES D'INDIVIDUS DIABETIQUES DE TYPE 2

Primary defects in lipolysis and insulin action in skeletal muscle cells from type 2 diabetic individuals

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Primary defects in lipolysis and insulin action in skeletal muscle cells from type 2 diabetic individuals

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ABSTRACT

A decrease in skeletal muscle lipolysis and hormone sensitive-lipase (HSL) expression has been linked to insulin resistance in obesity. The purpose of this study was to identify potential intrinsic defects in lipid turnover and lipolysis in myotubes established from obese and type 2 diabetic subjects. Lipid trafficking and lipolysis were measured by pulse-chase assay with radiolabeled substrates in myotubes from non-obese/non-diabetic (lean), obese/non-diabetic (obese) and obese/diabetic (T2D) subjects. Lipolytic protein content and level of Akt phosphorylation were measured by Western blot. HSL was overexpressed by adenovirus-mediated gene delivery. Myotubes established from obese and T2D subjects had lower lipolysis (−30–40%) when compared to lean, using oleic acid as precursor. Similar observations were also seen for labelled glycerol. Incorporation of oleic acid into diacylglycerol (DAG) and free fatty acid (FFA) level was lower in T2D myotubes, and acetate incorporation into FFA and complex lipids was also lower in obese and/or T2D subjects. Both protein expression of HSL (but not ATGL) and changes in DAG during lipolysis were markedly lower in cells from obese and T2D when compared to lean subjects. Insulin-stimulated glycogen synthesis (−60%) and Akt phosphorylation (−90%) were lower in myotubes from T2D, however, overexpression of HSL in T2D myotubes did not rescue the diabetic phenotype. In conclusion, intrinsic defects in lipolysis and HSL expression co-exist with reduced insulin action in myotubes from obese T2D subjects. Despite reductions in intramyocellular lipolysis and HSL expression, overexpression of HSL did not rescue defects in insulin action in skeletal myotubes from obese T2D subjects.

1. Introduction

Type 2 diabetes (T2D) is a metabolic disorder characterized with chronic hyperglycemia that affects the way the body utilizes energy. It is initiated by a combination of factors, including defects in regulation of glucose homeostasis and insulin resistance, a condition in which the body's skeletal muscle, adipose and liver tissue do not respond effectively to insulin [1]. Insulin resistance is possibly partly induced by chronic lipid overload in skeletal muscle, especially caused by long-chain acyl-CoAs, diacylglycerols (DAG) and ceramides [2–4].

Lipids are stored as triacylglycerols (TAG) in lipid droplets within skeletal muscle, called intramuscular triacylglycerol (IMTG), and upon energy demand e.g. during exercise, IMTG is used as energy source by healthy subjects [5,6]. There are evidences that increased IMTG is associated with higher levels of lipotoxic intermediates such as DAG and ceramides that might inhibit insulin signalling [7]. However, the mechanism by which IMTGs might contribute to lipotoxicity in obese, insulin resistant, or T2D subjects, remains poorly understood. Recent data suggest that intramyocellular dynamics, like lipid influx and altered rate of lipid turnover, may play an important role in developing insulin resistance [8]. Lipid turnover has a significant impact on insulin sensitivity and glucose homeostasis. Skeletal muscle tissue lipid oxidation and fatty acid (FA) incorporation into TAG are altered in obese individuals with T2D compared to BMI-matched controls, but only the disturbances in TAG incorporation are conserved in cultured myotubes [9–11].

TAG breakdown is mediated by lipases. The first step in hydrolysis of TAGs in skeletal muscle is catalysed by adipose triglyceride lipase (ATGL) [12]. Monoacylglycerol (MAG) lipase (MGL) and hormone-

Abbreviations: ACSL, acyl-CoA synthetase; ATGL, adipose triglyceride lipase; CE, cholesteryl esters; DAG, diacylglycerol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GIR, glucose infusion rate; HSL, hormone sensitive lipase; IMTG, intramuscular triacylglycerol; MGL, monoacylglycerol (MAG) lipase; OA, oleic acid; PL, phospholipids; T2D, type 2 diabetes; TAG, triacylglycerols

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sensitive lipase (HSL) were the first lipases identified, and both are highly expressed in skeletal muscle. HSL displays a 10-fold higher specificity for DAG compared to TAG, MAG and cholesteryl esters (CE) [13–15]. Recently, we and others have observed that the expression of ATGL and HSL seems to be altered in myotubes from obese and obese type 2 diabetic individuals when compared to lean controls, however the results are inconsistent. In short, protein expression of ATGL and HSL has been reported to be unaltered or reduced in myotubes from obese and T2D subjects [12,16–21]. Of interest, a selective pharmacological inhibition of lipolysis in myotubes from lean healthy donors was sufficient to inhibit insulin action [16]. The molecular mechanism involves at least in part DAG-mediated protein kinase C (PKC) activation [16]. We therefore hypothesized that reduced muscle HSL protein content could contribute to obesity-related insulin resistance. Because primary human muscle cells retain some of the phenotypic characteristic of their donors [10,22,23], we aimed to identify potential intrinsic defects in lipolysis and HSL expression in myotubes established from obese and obese T2D compared to lean subjects. We further determined whether overexpression of HSL could rescue the insulin resistant phenotype of myotubes from T2D subjects.

2. Material and methods

2.1. Materials

Dulbecco's modified Eagles medium (DMEM–Glutamax™), DMEM without phenol red, heat-inactivated foetal calf serum (FCS), αMEM, human epithelial growth factor (hEGF), fetuin, gentamycin, and penicillin–streptomycin and amphotericin B were purchased from Gibco Invitrogen (Gibco, Life Technologies, Paisley, UK). Ultroser G was purchased from PALL Life Science (Port Washington, NY, US), insulin (Actrapid®) from NovoNordisk (Bagsvaerd, Denmark), BSA (bovine serum albumin) (essentially fatty acid-free), L-carnitine, Dulbecco's phosphate-buffered saline (DPBS with Mg^{2+} and Ca^{2+}), oleic acid (OA, 18:1, n-9), glycerol, triacsin C, HEPES, extracellular matrix (ECM) gel, glycogen, dexamethasone, protease inhibitor and phosphatase I and II inhibitors, were all obtained from Sigma-Aldrich (St Louis, MO, US). [1-^{14}C]oleic acid (58.2 mCi/mmol), [1-^{14}C]acetate (56.0 mCi/mmol), [^{14}C(U)]glycerol (142 mCi/mmol) and D[^{14}C(U)]glucose (2.9 mCi/mmol) were from PerkinElmer NEN® (Boston, MA, US). Corning CellBIND® tissue culture plates (96- and 12-well plates) were obtained from Corning Life-Sciences (Schiphol-Rijk, The Netherlands). Isoplate® scintillation plates and OptiPhase Supermix, and all liquid scintillations were performed by the 1450 MicroBeta TriLux scintillation or Packard Tri-Carb 1600 counters, were obtained from PerkinElmer (Shelton, CT, US). Thin layer chromatography plates were purchased from Merck (Darmstadt, Germany), nitrocellulose membrane from Hybond ECL (Amersham Biosciences, Boston, MA, US) and chemiluminescence reagent and hyperfilm ECL from GE Healthcare. Antibodies for pAkt Ser473 (#4060), Akt (#4691), ATGL (#2138), HSL (#4107) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, #2118) was purchased from Cell Signalling Technology (Beverly, MA, US). Protein assay reagent was purchased from BioRad (Copenhagen, Denmark) or Pierce™ BCA protein assay kit (Thermo Scientific, Rockford, IL). Human HSL cDNA was cloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA) and obtained from Vector Biolabs (Philadelphia, PA). All other chemicals used were of standard commercial high-purity quality.

2.2. Human study subjects

Eight non-obese/non-diabetic (lean) control subjects, nine obese/non-diabetic (obese) subjects and eight obese/diabetic (T2D) subjects participated in the study (Table 1) [24]. Only sedentary subjects were recruited. The diagnosis of type 2 diabetes was based on fasting plasma glucose ≥7.0 mmol/L, HbA1c ≥ 6.5% and/or use of one or more antidiabetic drug. Diabetic patients were treated either with diet alone or in combination with sulfonylurea, metformin or insulin, which was withdrawn 1 week before the study. The patients had no diabetic complications apart from simplex retinopathy that was self-reported based on previous diagnosis by an ophthalmologist. The control subjects had normal fasting glucose concentrations and HbA1c levels and no family history for type 2 diabetes. The groups were matched with respect to age, but differed by BMI, fasting plasma glucose concentrations, fasting serum insulin levels, HbA1c and glucose infusion rate by hyperinsulinemic euglycemic clamp (HEC, 40 mU/m^2 per min of insulin, after overnight fasting for 10 h). Muscle biopsies were obtained from *m. vastus lateralis* in the fasted state by needle biopsy under local anaesthesia. All subjects gave written informed consent, and the local ethics committee of Funen and Vejle County approved the study. Cell cultures were established from proliferated satellite cells as previously described [25].

Table 1
Clinical characteristics of the biopsy donors.

Clinical variables	Lean	Obese	T2D
Age (years)	51 ± 3.5	47 ± 4.5	50 ± 4.8
Body mass index (kg/m^2)	24 ± 1.8	34 ± 5.0*	33 ± 3.8*
Fasting plasma glucose (mmol/L)	5.7 ± 0.4	5.7 ± 0.6	10 ± 2.1*#
Fasting serum insulin (pmol/L)	25 ± 20	57 ± 16*	97 ± 33*#
HbA1c (%)	5.6 ± 0.2	5.4 ± 0.3	7.6 ± 1.5*#
Glucose infusion rate (mg/min/m^2)	392 ± 64	235 ± 64*	121 ± 61*#

Values represent means ± SD (n = 8–9 per group).
* $p < 0.05$ vs lean.
\# $p < 0.05$ vs obese. T2D, type 2 diabetes (Bonferroni adjusted).

2.3. Cell culture

Myoblasts from control, obese and T2D subjects were cultured on multi-well plates or 25 cm^2 flasks in DMEM–Glutamax™ (5.5 mmol/L glucose), 2% FCS, 2% Ultroser G, 25 IU penicillin, 25 μg/mL streptomycin, and 1.25 μg/mL amphotericin B or in DMEM–Glutamax™ (5.5 mmol/L glucose) supplemented with 10% FCS, 10 ng/mL hEGF, 0.39 μg/mL dexamethasone, 0.05% BSA, 0.5 mg/mL fetuin, 50 ng/mL gentamycin and 50 ng/mL amphotericin B. At 70–80% confluence, the growth medium was replaced by DMEM–Glutamax™ supplemented with 2% FCS, 25 IU penicillin, 25 μg/mL streptomycin, 1.25 μg/mL amphotericin B, and 25 pmol/L insulin or αMEM supplemented with 2% FCS, 0.5 mg/mL fetuin, 25 IU penicillin and 25 μg/mL streptomycin to induce differentiation. The cells were cultured in humidified 5% CO_2 atmosphere at 37 °C, and the media were changed every 2–3 days. Human myotubes were allowed to differentiate at a physiological concentration of insulin (25 pmol/L) and glucose (5.5 mmol/L).

2.4. Pulse–chase assay and lipid distribution from oleic acid

Myotubes were cultured on 96-well or 12-well plates. On day six of differentiation the myotubes were pulsed with [1-^{14}C]oleic acid (OA, 100 μmol/L, 0.5 μCi/mL) or [^{14}C(U)]glycerol (10 μmol/L, 0.5 μCi/mL) for 24 h in differentiation medium. After pre-labelling, the cells were washed twice with 0.5% fatty acid-free BSA in DPBS at 37 °C. Some of the OA-labelled cells were harvested at the end of the pulse period (T0) with two additions of 125 μL distilled water to determine OA incorporation into TAG, DAG, FFA (free fatty acid), CE (cholesteryl esters) and PL (phospholipids). Following the pulse, myotubes were chased for 3 h with DPBS-medium containing 10 mmol/L HEPES, 100 μmol/L glucose and 0.5% fatty acid-free BSA. After 3 h cell-associated radioactivity for [^{14}C(U)]glycerol was determined, and lipid distribution of [1-^{14}C]OA was measured. Lipolysis was measured as [1-^{14}C]OA and [^{14}C(U)]glycerol released from the cells. Re-esterification of OA was calculated as total lipolysis (w/triacsin C, 10 μmol/L) minus basal lipolysis (wo/triacsin C) after 3 h [26]. Triacsin

C inhibits long-chain fatty acyl-CoA synthetase (ACSL) and will therefore inhibit, among other pathways, fatty acid oxidation and re-esterification of fatty acids into the TAG pool. Cellular uptake of radiolabel glycerol was estimated as sum of cell-associated glycerol plus total lipolysis, while uptake of oleic acid was calculated as sum of total lipids (TAG, DAG, FFA, CE and PL) at the end of the chase period (T3) plus total lipolysis. Fractional lipolysis was calculated as total lipolysis/uptake. For lipid distribution, cellular lipids were extracted as described earlier [11]. Briefly, homogenized cell fractions were extracted, lipids were separated by thin layer chromatography (TLC) using hexane-diethylether-acetic acid (65:35:1, v/v/v) as developing solvent. The radioactivity was quantified by liquid scintillation. Finally, the protein content of each sample was determined by use of Coomassie reagent and results standardized according to this value for each well [27].

2.5. Lipid distribution from labelled acetate

Myotubes were cultured on 12-well plates coated with ECM gel and incubated with [1-^{14}C]acetate (2 μCi/mL, 100 μmol/L) for 4 h on day 7 of differentiation, to study lipid distribution from labelled acetate. Myotubes were placed on ice, washed three times with PBS (1 mL), harvested into a tube with two additions of 125 μL distilled water and frozen at −20 °C. The cells were later assayed for protein [27], and cellular lipids were extracted [11]. Briefly, the homogenized cell fraction was extracted, lipids were separated by TLC and the radioactivity was quantified by liquid scintillation. Another non-polar solvent mixture of hexane-diethylether-acetic acid (50:50:1, v/v/v) followed by pure hexane was used to separate free cholesterol from diacylglycerol (DAG). The amount of neutral lipids was calculated by using total protein levels for standardization.

2.6. Western blot analysis

Myotubes were harvested in a buffer containing 50 mmol/L HEPES, pH 7.4, 2 mmol/L EDTA, 150 mmol/L NaCl, 30 mmol/L $NaPO_4$, 10 mmol/L NaF, 1% Triton X-100, 10 μL/mL protease inhibitor, 10 μL/mL phosphatase I inhibitor, 10 μL/mL phosphatase II inhibitor, and 1.5 mg/mL benzamidine HCl. Cell extracts were sonicated and stored at −80 °C. Solubilized proteins (30 μg) were run on a 4–20% SDS–PAGE, transferred onto nitrocellulose membrane and incubated with the primary antibodies: ATGL, HSL, pAkt Ser473, and Akt. Subsequently, immunoreactive proteins were visualized using the ChemiDoc MP Imaging System and data analysed using the Image Lab 4.1 version software. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control.

2.7. Overexpression of HSL

Human HSL cDNA was cloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA, US). DNA sequencing was performed to check correct insertion of the cDNA using an ABI3100 automatic sequencer. An adenovirus expressing in tandem green fluorescent protein (GFP) and HSL was constructed, purified, and titrated (Vector Biolabs, Philadelphia, PA, US). An adenovirus containing the GFP gene only was used as a control. Myotubes were infected with the control (GFP), and HSL adenovirus at day 4 of differentiation and remained exposed to the virus for 24 h in serum-free DMEM containing 100 μmol/L of oleate complexed to BSA (ratio 4:1). No adenovirus-induced cellular toxicity was observed as determined by chemiluminescent quantification of adenylate kinase activity. For insulin signalling experiments, infected myotubes were preincubated with a glucose- and serum-free DMEM for 90 min and then incubated for 20 min in αMEM (5.5 mmol/L glucose) with or without 100 nmol/L of insulin.

2.8. Determination of glycogen synthesis

Cells were preincubated with a glucose- and serum-free DMEM for 90 min and then exposed to DMEM supplemented with D[^{14}C(U)]glucose (1 μCi/mL, 5.5 mmol/L) in the presence or absence of 100 nmol/L insulin for 3 h. In preliminary unpublished studies from our laboratory, we have seen a defective insulin-stimulated glycogen synthesis at all concentrations of insulin ranging from 1 to 100 nM. Based on this, we decided to use 100 nM of insulin for experiment to reach maximal stimulation of glycogen synthesis in all experiments and type of donors. After incubation, glycogen synthesis was determined as described previously [28].

2.9. Presentation of data and statistics

All values are reported as means ± SEM, except from clinical values presented in Table 1 that is reported as means ± SD. The value n usually represents the number of different donors used, each with at least triplicate samples. Statistical analyses were performed using either GraphPad Prism 5.0 for Windows (GraphPad Software Inc., San Diego, CA, US) or IBM® SPSS® (version 21, IBM Corporation, New York, NY, US). Two-tailed unpaired Student's t-tests were performed to determine the difference between myotubes from two donor groups. Linear mixed models (LMMs) were used to compare effects of different treatments used in accumulation and lipolysis experiments for labelled glycerol between all donor groups (IBM SPSS). The linear mixed models include all observations in the statistical analyses and at the same time take into account that not all observations are independent. Correlations are presented as Spearman's correlation coefficient (r). Clinical data were compared using ANOVA with Bonferroni adjustment for multiple comparisons (IBM SPSS). A p-value < 0.05 was considered significant.

3. Results

3.1. Clinical characteristics of the biopsy donors

Fasting plasma glucose, serum insulin and HbA1c (Table 1) were significantly higher in obese/diabetic subjects (T2D) than in obese/non-diabetic subjects (obese) with similar body mass index (BMI), whereas glucose infusion rate (GIR) was lower in T2D subjects. As expected, clinical characteristics of non-obese/non-diabetic subjects (lean) were different from obese subjects with respect to BMI, fasting serum insulin and GIR. The age was not significantly different between the three donor groups.

3.2. Total lipolysis was lower in myotubes from obese and type 2 diabetic subjects, while lipid distribution was altered in myotubes from type 2 diabetic subjects

To identify intrinsic defects in lipid metabolism in myotubes from obese and T2D subjects we first assessed uptake, synthesis, and hydrolysis of fatty acids using [1-^{14}C]oleic acid (OA). Cellular uptake of OA was unchanged (Fig. 1A), while a lowering in total lipolysis (w/triacsin C) in myotubes from obese (−30%) and T2D subjects (−40%) compared to myotubes from lean subjects (Fig. 1B). Related to uptake of labelled precursor, fractional lipolysis (total lipolysis/total uptake) was significantly lower in myotubes from T2D subjects (−25%) compared to lean subjects (Fig. 1C). Re-esterification (measured as total lipolysis minus basal lipolysis) (wo/triacsin C) of OA was not different between donor groups (data not shown). Lipid distribution was altered in myotubes from T2D subjects after 24 h incubation with radiolabelled OA; free fatty acids (FFA) and diacylglycerol (DAG) were lower in cells from the T2D group, while triacylglycerol (TAG), phospholipids (PL) and cholesteryl esters (CE) were unchanged (Fig. 1D).

To verify the reduced lipolysis of labelled OA observed in myotubes from obese and T2D subjects with another substrate in glycerolipid metabolism, [^{14}C(U)]glycerol was used to label the lipid pool. There was a

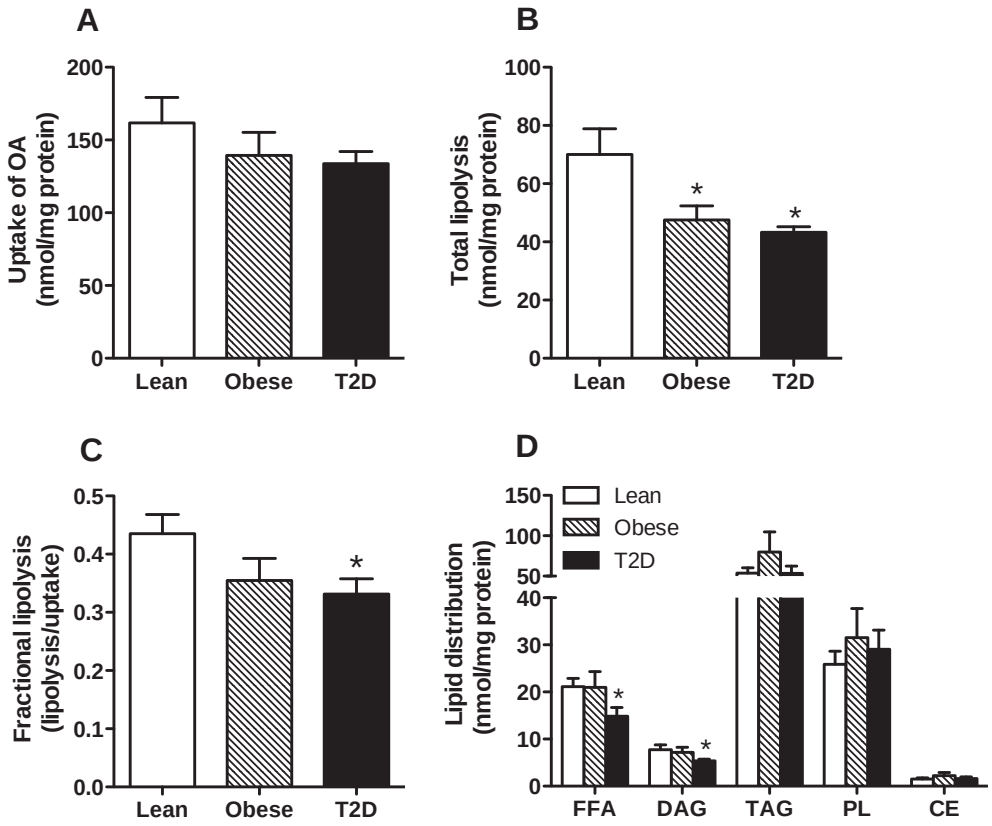

Fig. 1. Lipolysis and lipid distribution in human myotubes. Human myotubes were pretreated with [1-^{14}C]oleic acid (OA) (100 μmol/L, 0.5 μCi/mL) for 24 h. After another 3 h, total lipolysis (w/triacsin C, 10 μmol/L) was measured as [1-^{14}C]OA released from the cells. The figure shows uptake of OA (sum of total cellular lipids plus total lipolysis) (A), total lipolysis, (B) fractional lipolysis (C) and lipid distribution of free fatty acids (FFA), diacylglycerol (DAG), triacylglycerol (TAG), phospholipids (PL) and cholesteryl esters (CE) from labelled OA. Results represent means ± SEM (n = 6). *p < 0.05 vs lean.

50% lower cellular uptake (Fig. 2A) of glycerol and lower total lipolysis (w/triacsin C) in myotubes from both obese (−60%) and T2D (−75%) subjects compared to myotubes from lean subjects (Fig. 2B). Fractional lipolysis was significantly lower in myotubes from T2D subjects (−20%) than in cells from lean subjects (Fig. 2C).

3.3. Lipogenesis and lipid synthesis from acetate was reduced in myotubes from obese and type 2 diabetic subjects

We also studied how the cells from the different donor groups managed lipogenesis and synthesis of complex lipids from [1-^{14}C]acetate and whether this process also differed between donor groups as seen for fatty acid and glycerol uptake and lipolysis (Figs. 1 and 2). Lipogenesis was significantly lower in myotubes from obese and T2D subjects for DAG, phospholipids (PL), CE and total lipids compared to myotubes from lean subjects (Fig. 3). FFA was only significantly lower in myotubes from T2D subjects than in myotubes from lean subjects, while TAG was unchanged.

3.4. Protein expression of HSL and levels of DAG were lower in myotubes from obese subjects

Recently, it has been shown that protein expression of HSL was reduced in muscle biopsies from obese subjects [16]. We wanted to

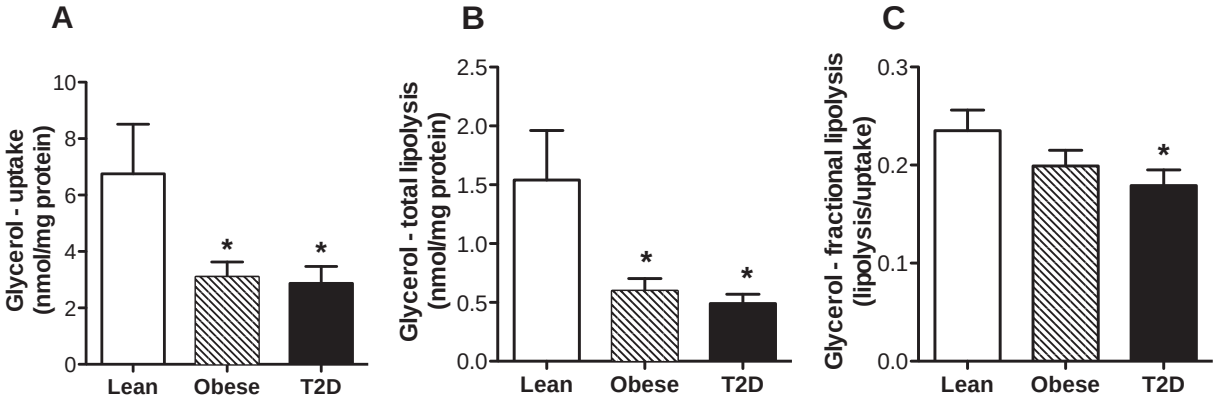

Fig. 2. Glycerol uptake and lipolysis in human myotubes. Human myotubes were pretreated with [^{14}C(U)]glycerol (10 μmol/L, 0.5 μCi/mL) for 24 h. After another 3 h, total lipolysis (w/triacsin C, 10 μmol/L) was measured as [^{14}C(U)]glycerol released from the cells and cell-associated [^{14}C(U)]glycerol was measured. The figure shows uptake (sum of cell-associated glycerol plus total lipolysis) (A), total lipolysis (B) and fractional lipolysis (C). Results represent means ± SEM (n = 6). *p < 0.05 vs lean (Bonferroni adjusted).

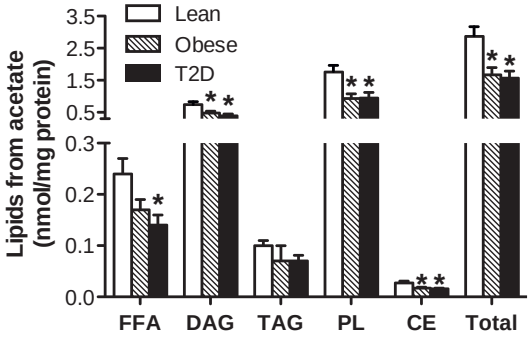

Fig. 3. Lipogenesis and lipid distribution from acetate in human myotubes. Human myotubes were incubated with [1-^{14}C]acetate (100 μmol/L, 2 μCi/mL) for 4 h. Lipid distribution was measured by thin-layer chromatography. The figure shows synthesis of free fatty acid (FFA), diacylglycerol (DAG), triacylglycerol (TAG), phospholipids (PL), cholesteryl ester (CE) and total lipogenesis (sum of FFA, DAG, TAG and CE). Results represent means ± SEM (n = 7). *p < 0.05 vs lean.

explore whether this reduction was conserved in myotubes from obese and T2D in vitro. The HSL protein expression was lower in myotubes from obese (−30%) and obese T2D (−60%) subjects, when compared to myotubes from lean subjects. The protein expression of ATGL was not different between the donor groups (Fig. 4).

Further, the concentration of OA-labelled DAG during lipolysis was markedly higher in myotubes from lean subjects compared to both obese (−75%) and T2D (−90%) (Fig. 5A). Concentration of intracellular FFA was only lower in T2D cells during lipolysis when compared to myotubes from lean (Fig. 5B). Levels of TAG were not changed during lipolysis under the experimental conditions used in this study (data not shown).

3.5. Turnover of DAG during lipolysis in myotubes correlated with BMI, insulin, plasma glucose and GIR

For all donors, there were negative correlations between turnover of DAG during lipolysis and BMI (Fig. 6A), fasted plasma insulin (Fig. 6B) and fasted plasma glucose levels (Fig. 6C), while there was a positive correlation between changes of DAG during lipolysis and glucose infusion rate (GIR) (Fig. 6D). We obtained similar results when correlating the same parameters to levels of FFA after pulse–chase treatment with labelled OA (data not shown).

3.6. Overexpression of HSL in type 2 diabetic myotubes did not rescue the cells from insulin resistance

To evaluate whether the diabetic phenotype could be altered by overexpression of HSL in myotubes, we used an adenovirus gene delivery method to overexpress HSL in myotubes from T2D subjects that resulted in a 15-fold increase in the expression of HSL (Figs. 7A and B). HSL overexpression slightly reduced TAG content (Fig. 7C) in myotubes without altering baseline DAG content (Fig. 7D). Of importance, the T2D myotubes were less insulin responsive in terms of glycogen synthesis (Fig. 7E) and Akt phosphorylation (Fig. 7F) than lean control myotubes, confirming an intrinsic insulin resistance. Further, insulin resistance persisted in T2D myotubes over-expressing HSL.

4. Discussion

This study shows for the first time a lower lipolysis rate of labelled oleic acid (OA) in myotubes established from obese and obese T2D subjects when compared to lean control cells. Protein expression of hormone-sensitive lipase (HSL) was lower in myotubes established from obese and T2D donors, and this was reflected by a lower turnover of diacylglycerol (DAG) during lipolysis for the obese groups compared to myotubes established from lean subjects. Insulin-stimulated glycogen synthesis and Akt-phosphorylation were strongly reduced in myotubes from T2D, when compared to lean subjects, confirming an insulin resistant state in these cells [23,29,30]. Overexpression of HSL in T2D myotubes was not sufficient to rescue insulin action and the diabetic phenotype. This suggests that intrinsic defects in insulin action and glycogen synthase activation persist in cultured myotubes independently of primary defects in lipid turnover and lipolysis.

Previous studies have shown that there are differences in handling of lipids and lipase protein content in skeletal muscle from lean, obese and T2D individuals [9,12,16], but no mechanistic studies have yet demonstrated functional defects in lipolysis in skeletal muscle cells from obese and T2D individuals. Our study confirms that there is an intrinsic difference in lipid handling between myotubes from lean, obese and T2D subjects. We observed that the uptake of glycerol was different in obese and T2D cells compared to control myotubes and also incorporation of OA and acetate into free fatty acids (FFA) and complex lipids, suggesting that this difference is connected to the synthesis, uptake and esterification of fatty acids. Although de novo lipogenesis in myotubes seem to be quite low, this pathway has previously been shown to be functional [31,32]. Despite marginal differences in the uptake and/or esterification of lipolysis metabolites between donors, we observed that lipolysis was specifically lower in myotubes from T2D compared to lean subjects after adjusting for differences in uptake of OA and glycerol (fractional lipolysis). Altogether, our data show that primary defects in lipid handling and turnover persist in vitro in myotubes established from obese subjects with and without T2D.

A recent work by Sparks et al. [9] showed that incorporation of FFAs into TAG was lower in myotubes from T2D subjects, with a correlation of TAG level/incorporation between muscle tissue and primary myotubes. Further, myotubes from T2D subjects accumulated more lipids when activating lipid metabolism through LXR than control cells [33]. These studies indicate that the ability to incorporate FFAs into TAG and complex lipids is an intrinsic feature of human skeletal muscle

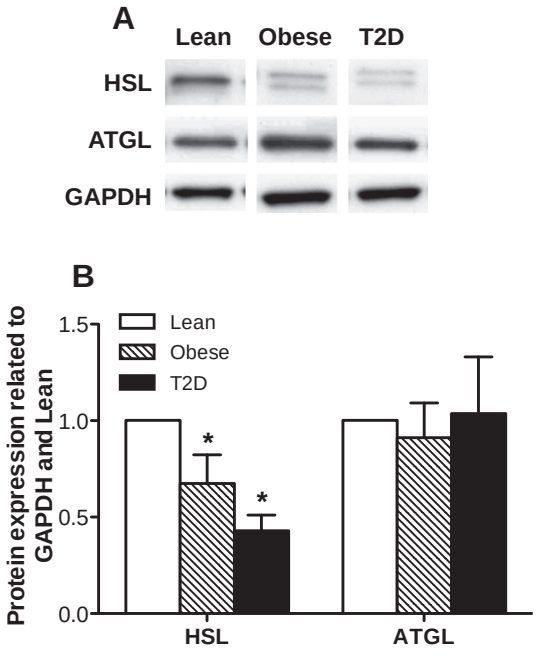

Fig. 4. Protein expressions of lipases. Protein expression of hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) in myotubes was studied and related to GAPDH. Representative Western blots are shown in (A). Values in (B) represent fold change of proteins in myotubes from obese and T2D relative to lean, given as means ± SEM (n = 8). *p < 0.05 vs lean.

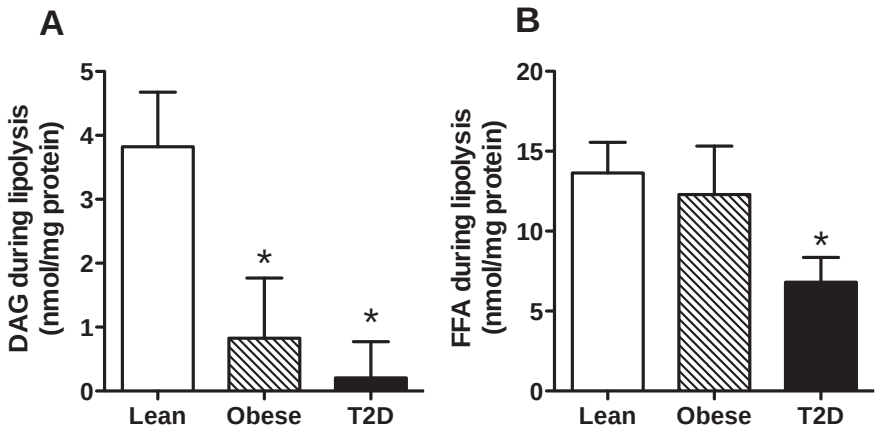

Fig. 5. Changes in diacylglycerol and free fatty acid levels during lipolysis. Human myotubes were pretreated with [1-^{14}C]oleic acid (OA) (100 μmol/L, 0.5 μCi/mL) for 24 h. After another 3 h, lipid distribution of [1-^{14}C]OA and total lipolysis (w/triacsin C) as [1-^{14}C]OA released from the cells, were determined. The figure shows change in levels of diacylglycerol (DAG) (A) and change in free fatty acids (FFA) (B) during lipolysis. Results represent means ± SEM (n = 6). $^{*}p < 0.05$ vs lean.

cells that is different in myotubes from individuals with T2D. We observed that lipolysis was lower in myotubes derived from both obese groups when compared to the lean group. Incorporation of OA into DAG was lower only in the T2D group (Fig. 1D), but the turnover of the same fatty acids during lipolysis was markedly different between lean and both obese groups (Fig. 5A). Previous studies suggested that dysregulated lipolysis could increase toxic lipid intermediate levels in skeletal muscle, leading to the development of insulin resistance [8,14,34]. One could speculate whether the observed changes in lipolysis found in this study, is a sign of difference in lipid turnover in the various donor groups. Also incorporation of acetate into FFAs and complex lipids (de novo lipogenesis) was lower in myotubes from both obese groups

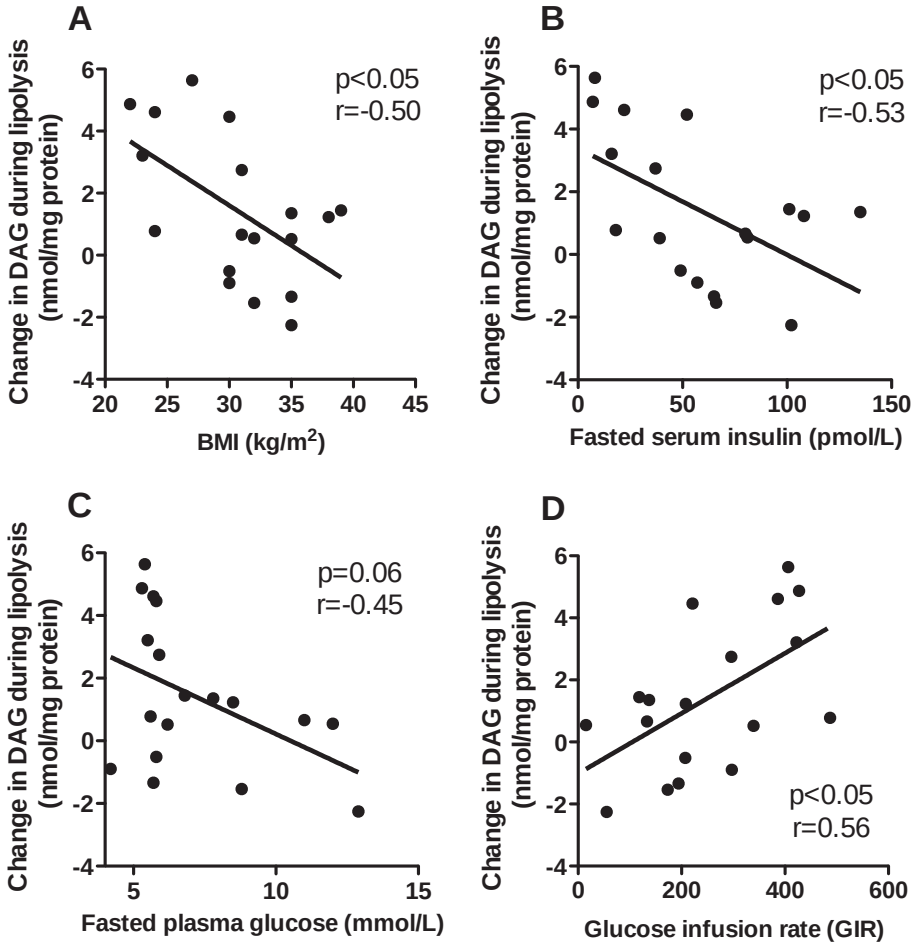

Fig. 6. Correlations of changes in diacylglycerol during lipolysis vs clinical metabolic phenotypes. The relationships between change in diacylglycerol (DAG) during lipolysis in vitro and corresponding clinical data including body mass index (BMI) (A), fasted insulin (B), fasted plasma glucose (C) and glucose infusion rate (D) were determined by non-parametric correlations (Spearman). n = 6 donors in each group.

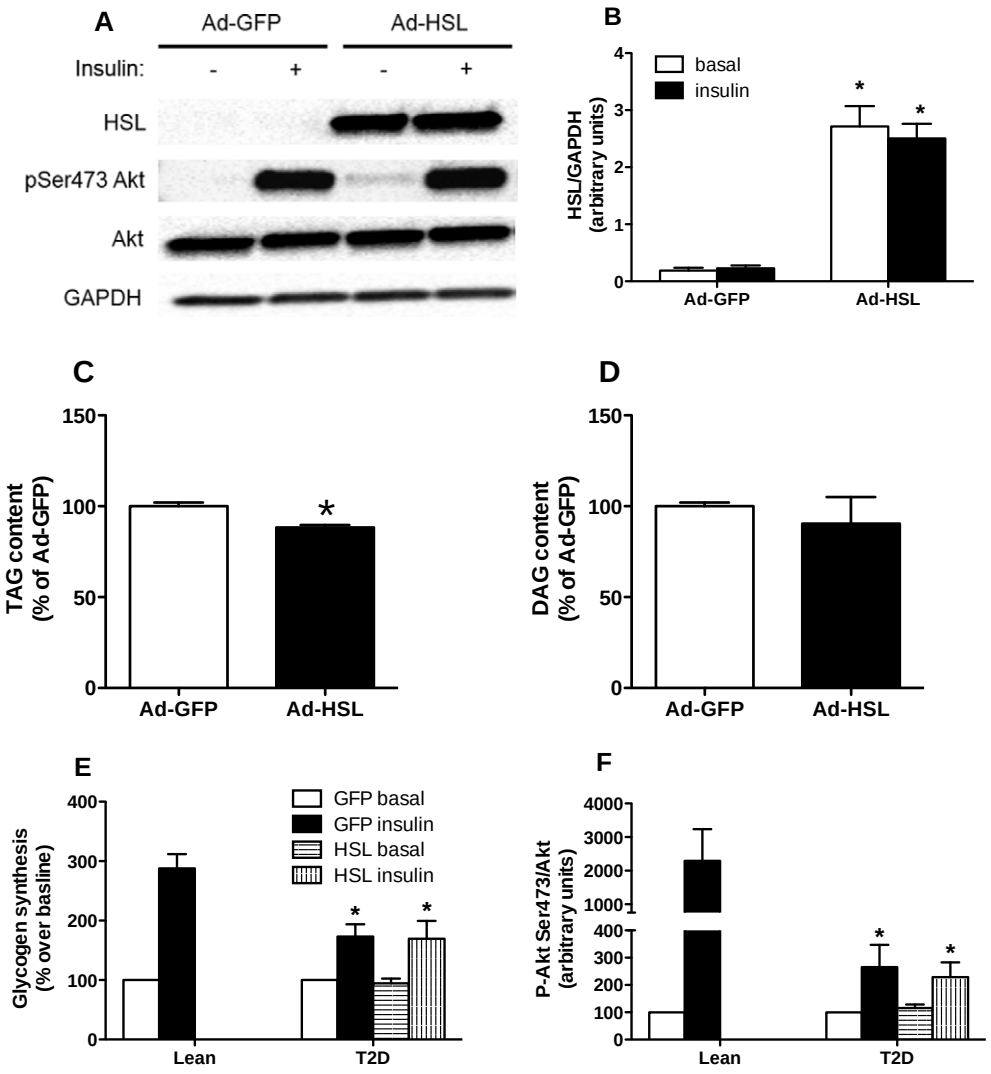

Fig. 7. The effect of HSL overexpression on insulin sensitivity markers in myotubes. An adenovirus gene delivery method was used to overexpress HSL in myotubes as shown in (A) and (B). Incorporation of [1-^{14}C]oleic acid (OA) (100 μmol/L, 0.5 μCi/mL) into triacylglycerol (TAG) (C) and diacylglycerol (DAG) (D) was measured after 18 h. Glycogen synthesis (E) was measured as D[^{14}C(U)]glucose (1 μCi/mL, 5.5 mmol/L) incorporation into glycogen in the presence or absence of 100 nmol/L insulin for 3 h, (n = 4 donors per group). Quantitative bar graph from Western blots of Ser473 phosphorylation of Akt (F) in the presence or absence of 100 nmol/L insulin for 20 min (n = 6–8). *p < 0.05 vs lean.

than myotubes from lean group, suggesting that the difference in handling of complex lipids may in large part affect lipid metabolism. Previous studies performed on human skeletal muscle cells failed to reveal significant differences in lipid handling between non-diabetic and diabetic myotubes [10,11]. Further, we observed a negative correlation between in vitro turnover of DAG during lipolysis and body mass index, and a positive relationship between in vitro turnover of DAG during lipolysis and whole-body insulin sensitivity (GIR), thus indicating that lower lipid turnover and lipolysis is an intrinsic characteristic of skeletal muscle cells in the context of obesity and/or insulin resistance. This is in line with the observation that low lipid turnover in skeletal muscle is related to lipotoxicity and insulin responsiveness in obesity, while high lipid turnover as observed in endurance-trained individuals may have a protective effect [3,14].

Badin et al. [16] hypothesized that an imbalance of ATGL relative to HSL (expression or activity) may contribute to DAG accumulation and insulin resistance. They found that overexpression of ATGL reduced insulin signalling in cells that muscle HSL protein was reduced in obese subjects and that HSL overexpression could restore a proper lipolytic balance in ATGL overexpressed cells. In the Badin study however, no difference in HSL protein expression in biopsies from T2D subjects was observed likely because the T2D group was older and not well matched to the obese control group. Further, Jocken et al. [12] have shown in vivo that HSL protein content was lower in biopsies from skeletal muscle of obese men, while ATGL protein content was higher when compared to age-matched non diabetic lean controls. This difference in lipase content was accompanied by a lower ratio of DAG to TAG hydrolase activity in the obese men, reflecting functional defects in lipolysis. In this study, we observed a reduced protein level of HSL together with reduced total lipolysis and a corresponding change in levels of DAG and FFA during lipolysis in our in vitro myotube model and no alteration of ATGL protein expression, reflecting that functional defects in HSL expression and lipolysis are intrinsically retained in vitro in skeletal muscle cells in the context of obesity/T2D. Although down-regulation of HSL activity/expression has been linked to insulin resistance in human myotubes [16], overexpression of HSL in T2D cells was not sufficient to rescue insulin action and the diabetic phenotype in this study. However, we cannot rule out that increasing HSL expression may rescue insulin signalling and action in non-diabetic insulin resistant myotubes and/or exert a protective effect against lipid-induced insulin resistance. This should be examined in future studies.

In summary, this study raises a number of important questions. First, it is yet unclear how functional defects in lipolysis and insulin resistance are being intrinsically retained/imprinted in vitro in cultured skeletal muscle cells. Second, although primary defects in lipolysis, HSL protein content and insulin action co-exist in cultured primary muscle cells from obese T2D donors, these biological events appear independent from each other. Further studies are needed to unravel the molecular mechanisms underlying these defects. It would be of interest to examine in future studies how environmental changes (diet, exercise, disease progression) drive metabolic imprinting and remodels the gene expression pattern of skeletal muscle cells.

Transparency document

The Transparency document associated with these articles can be found, in the version.

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PUBLICATION 7 : UNE ALTERATION DE LA SIGNALISATION MUSCULAIRE DES RECEPTEURS AUX PEPTIDES NATRIURETIQUES RELIE LE DIABETE DE TYPE 2 A L'OBESITE

Defective natriuretic peptide receptor signaling in skeletal muscle links obesity to type 2 diabetes

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Defective Natriuretic Peptide Receptor Signaling in Skeletal Muscle Links Obesity to Type 2 Diabetes

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Circulating natriuretic peptide (NP) levels are reduced in obesity and predict the risk of type 2 diabetes (T2D). Since skeletal muscle was recently shown as a key target tissue of NP, we aimed to investigate muscle NP receptor (NPR) expression in the context of obesity and T2D. Muscle NPRA correlated positively with whole-body insulin sensitivity in humans and was strikingly downregulated in obese subjects and recovered in response to diet-induced weight loss. In addition, muscle NP clearance receptor (NPRC) increased in individuals with impaired glucose tolerance and T2D. Similar results were found in obese diabetic mice. Although no acute effect of brain NP (BNP) on insulin sensitivity was observed in lean mice, chronic BNP infusion improved blood glucose control and insulin sensitivity in skeletal muscle of obese and diabetic mice. This occurred in parallel with a reduced lipotoxic pressure in skeletal muscle due to an upregulation of lipid oxidative capacity. In addition, chronic NP treatment in human primary myotubes increased lipid oxidation in a PGC1α-dependent manner and reduced palmitate-induced lipotoxicity. Collectively, our data show that activation of NPRA signaling in skeletal muscle is important for the maintenance of long-term insulin sensitivity and has the potential to treat obesity-related metabolic disorders.

Obesity is a major risk factor of type 2 diabetes (T2D) and cardiovascular diseases (1,2). Although multiple hypotheses have been proposed, the link between obesity and the risk of T2D is still poorly understood. Over the last decade, several large cohort studies reported an inverse association between plasma natriuretic peptide (NP) levels and BMI (3,4), and the risk of T2D (5,6). Therefore, dysregulation of the NP system, referred to as the "NP handicap," might be an important factor in the initiation and progression of metabolic dysfunction, making NPs potential candidates linking obesity and T2D (7–10).

NPs, including atrial NP (ANP) and brain NP (BNP), are mainly known as heart hormones secreted in response to cardiac overload and mechanical stretch in order to regulate blood volume and pressure (11,12). ANP and BNP classically bind to a biologically active receptor A (NP receptor A [NPRA]) that promotes cGMP signaling (13). They are also quickly cleared from the circulation and degraded through NP clearance receptor (NPRC). The NPRA-to-NPRC ratio therefore controls the biological activity of NP at the target tissue level (14).

Besides their well-documented role in the cardiovascular system, several studies revealed a metabolic role of NP (15,16). Pioneering studies demonstrated a potent lipolytic role of these peptides in human adipose tissue (17,18), and more recent studies indicated they may play a role in the "browning" of human white fat cells (19) as well as in favoring fat oxidative capacity in human skeletal muscle cells (20). The underlying mechanism involves activation of cGMP signaling, induction of PGC1α (peroxisome

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proliferator–activated receptor γ coactivator-1α), and enhancement of mitochondrial respiration. Together these studies argue for an important role of NP in the regulation of whole-body energy metabolism. The lipolytic effect of NP is absent in mice naturally expressing high levels of NPRC in adipose tissue (19,21). However mice overexpressing BNP are protected from diet-induced obesity and insulin resistance, which suggests that the protective effect of NP is achieved by targeting other metabolic tissues such as skeletal muscles (22). We therefore hypothesized that a downregulation of NPRA and/or an upregulation of NPRC in skeletal muscle could contribute to the "NP handicap" and provide a novel pathophysiological and mechanistic link between obesity and T2D.

In the current study, through a comprehensive set of experiments in humans, mouse models of obesity and T2D, and human primary skeletal muscle cells, we demonstrated a pathophysiological link between obesity-induced insulin resistance and T2D and defective skeletal muscle NPR signaling. In addition, increasing circulating BNP levels in diabetic and high-fat diet (HFD)–fed mice improved blood glucose control and insulin sensitivity. These effects were accompanied by improved muscle insulin signaling resulting from reduced lipotoxic lipid pressure and elevated lipid oxidative capacity.

RESEARCH DESIGN AND METHODS

Clinical Studies and Human Subjects

Muscle biopsy samples from lean, obese with normal glucose tolerance, obese with impaired glucose tolerance (IGT), and obese subjects with T2D were obtained from three independent clinical studies. Study 1 included nine young lean and nine young obese subjects (Fig. 1*A–D*) (23). Study 2 included four middle-aged obese subjects with T2D and six with IGT at baseline and in response to 12 weeks of calorie restriction to induce weight loss and improve metabolic health (Fig. 1*E* and *F*) (24). Study 3 included 21 subjects with normal glucose tolerance but a wide range of body fat (Supplementary Fig. 1) (25). The clinical characteristics of the subjects are summarized in Supplementary Table 1. All volunteers gave written informed consent and the protocol was approved by an institutional review board. Studies were performed according to the latest version of the Declaration of Helsinki and the Current International Conference on Harmonization guidelines. Samples of vastus lateralis weighing 60–100 mg were obtained by muscle biopsy using the Bergström technique, blotted, cleaned, and snap frozen in liquid nitrogen (26). Insulin sensitivity was measured by hyperinsulinemic-euglycemic clamp after an overnight fast (27). An intravenous catheter was placed in an antecubital vein for infusion

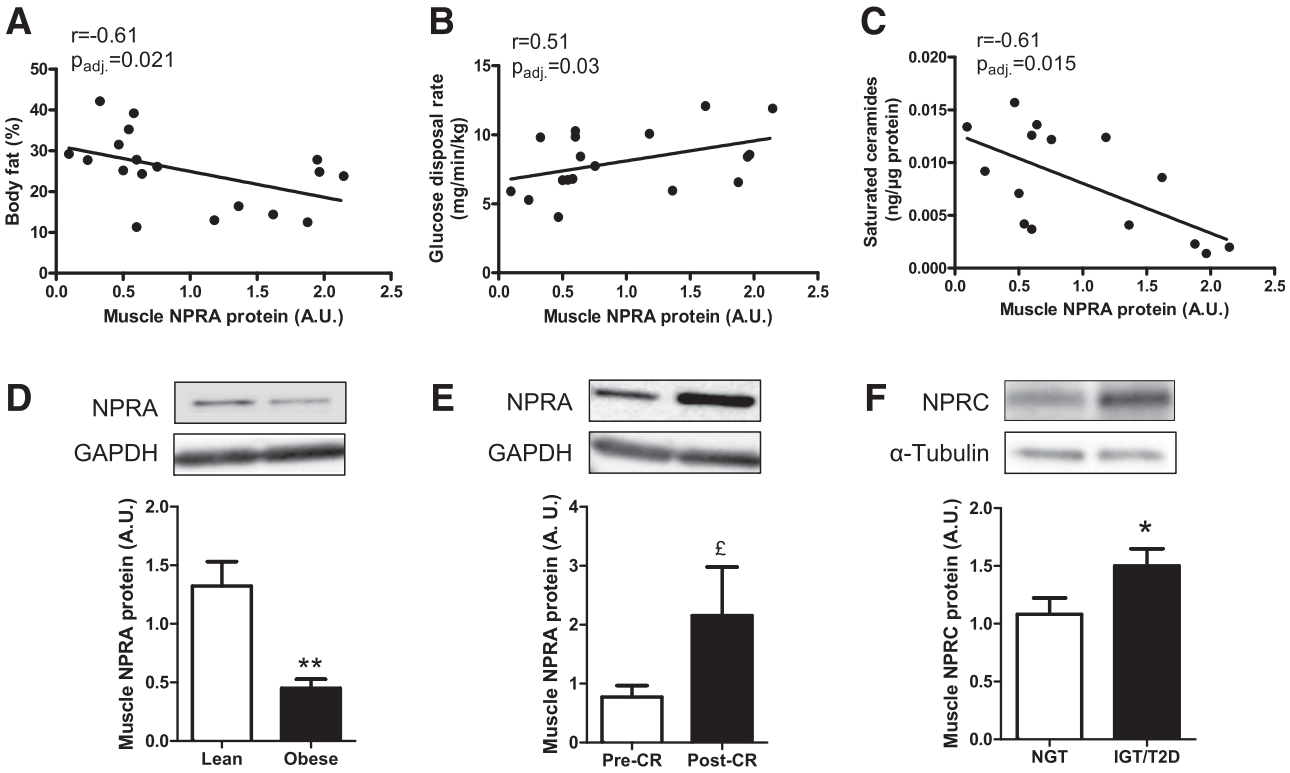

Figure 1—Skeletal muscle NPR expression relates to insulin sensitivity in humans. Correlation between vastus lateralis NPRA protein expression and percent body fat (*A*), glucose disposal rate measured by euglycemic-hyperinsulinemic clamp (*B*), and muscle-saturated ceramide content (*C*) (n = 15–20). *D*: NPRA protein levels in skeletal muscle of lean and obese subjects (*D*) and in obese subjects pre- and postcalorie restriction (pre-CR and post-CR) (*E*). *F*: NPRC protein levels in skeletal muscle of obese subjects with normal glucose tolerance (NGT) and with IGT and T2D. $^{**}P < 0.01$ vs. lean; £$P = 0.06$ vs. pre-CR; $^{*}P < 0.05$ vs. NGT (n = 6–10 per group).

of glucose and insulin during the clamp. A second catheter was placed retrograde in a dorsal vein of the contralateral hand for blood withdrawal. The hand was placed in a plastic heated box at ~60°C for arterialization of venous blood. Three blood samples were drawn before the initiation of insulin and glucose for the clamp and during the last 30 min of the clamp. A primed infusion of regular insulin (80 mU · min^{-1} · m^{-2}) was initiated and continued for 2 h. Plasma glucose was clamped at 90 mg/dL in all participants. Arterialized plasma glucose was measured at 5-min intervals and a variable infusion of exogenous glucose (20% solution) was given to maintain plasma glucose concentration. Plasma glucose was analyzed with a YSI 2300 STAT glucose analyzer (YSI Inc., Yellow Springs, OH), and plasma insulin was measured using an ultrasensitive ELISA kit (ALPCO Diagnostics, Salem, NH). Glucose disposal rate was adjusted by kilograms of fat-free mass. Body composition (considering a three-compartment model) was determined using a total-body dual-energy X-ray absorptiometer (DPX, Software 3.6; Lunar Radiation Corp., Madison, WI).

Mice and Diets

Five-week-old male diabetes-prone, obese *db/db* mice of the C57BL/KsJ-lept^{db}-lept^{db} strain with their nondiabetic lean littermate control *db/+* were used. For HFD studies, we used regular C57BL/6J male mice (JANVIER LABS). The mice were housed in a pathogen-free barrier facility (12-h light/dark cycle) with ad libitum access to water and food. After weaning, *db/db* and *db/+* mice were fed a normal chow diet (A04; SAFE Diets) for 4 weeks. C57BL/6J mice were fed for 16 weeks either a normal chow diet (10% energy as fat, D12450J; Research Diets, Inc., New Brunswick, NJ) or HFD containing 60% kcal from fat (D12492; Research Diets, Inc.). All experimental procedures were approved by a local institutional animal care and use committee and performed according to INSERM guidelines for the care and use of laboratory animals.

BNP Infusion Studies

Mice were randomly assigned to receive a saline vehicle (NaCl 0.9%) and/or chronic rat/mouse BNP1-32 (B9901; Sigma-Aldrich) at a rate of 5 or 10 ng/kg/min. Treatments were chronically administered intraperitoneally with mini-osmotic pumps (model 1004; Alzet, Cupertino, CA) (28). Mini-pumps were placed after 12 weeks of HFD and treatment was administered for 4 weeks in C57BL/6J mice and at 6 weeks of age in *db/db* mice. Body weight was measured weekly and body composition was assessed by quantitative nuclear magnetic resonance imaging (EchoMRI 3-in-1 system; Echo Medical Systems).

Glucose and Insulin Tolerance Tests

Six hour–fasted mice were injected intraperitoneally with a bolus of D-glucose at 2 g/kg (Sigma-Aldrich, Saint-Quentin Fallavier, France) and insulin 0.5 units/kg (Insuman Rapid; Sanofi, Paris, France) for glucose and insulin tolerance tests (GTT and ITT), respectively (29). Blood glucose levels were monitored from the tip of the tail with a glucometer (Accucheck; Roche, Meylan, France) at 0, 15, 30, 45, 60, and 90 min after injection. Radiolabeled GTTs were performed as previously described (29).

Blood Analyses and Tissue Collection

After an overnight fast, mice were decapitated and blood was collected into tubes containing EDTA and protease inhibitors. Organs and tissues were rapidly excised and snap frozen in liquid nitrogen before being stored at −80°C. Blood glucose was assayed using the glucose oxidase technique (Biomérieux, Paris, France), and plasma insulin was measured using an ultrasensitive ELISA kit (ALPCO Diagnostics). Plasma BNP was measured using the RayBio BNP Enzyme Immunoassay Kit (RayBiotech, Inc., Norcross, GA). HbA_{1c} and fructosamines were determined using a PENTRA 400 multianalyzer.

Human Skeletal Muscle Cell Culture

Satellite cells from rectus abdominis biopsies of healthy subjects with normal glucose tolerance (age 34.3 ± 2.5 years, BMI 26.0 ± 1.4 kg/m^2, fasting glucose 5.0 ± 0.2 mmol/L) were grown in DMEM supplemented with 10% FBS and growth factors (human epidermal growth factor, BSA, dexamethasone, gentamycin, fungizone, and fetuin) as previously described (23,30). Myotubes were differentiated up to 5 days and were treated with 100 nmol/L human ANP (A1663; Sigma-Aldrich) or BNP (B5900; Sigma-Aldrich) every day for the last 3 days.

Determination of Fatty Acid Metabolism

Pulse-chase experiments to determine lipolytic flux and oleate incorporation into total lipids, triacylglycerols (TAGs), and diacylglycerols (DAGs) by thin-layer chromatography were performed as previously described (31). Incorporation rates were normalized to total protein content in each well. Palmitate oxidation rates were measured as described previously (25).

Lipid Intermediate Determination

TAG and DAG content were measured by gas chromatography and ceramide and sphingomyelin species by high-performance liquid chromatography–mass spectrometry after total lipid extraction as previously described for mouse and human muscle tissues (25,29).

Western Blot Analysis

Soleus and gastrocnemius skeletal muscles, white and brown adipose tissues, and myotubes were homogenized in a buffer containing 50 mmol/L HEPES, pH 7.4, 2 mmol/L EDTA, 150 mmol/L NaCl, 30 mmol/L $NaPO_4$, 10 mmol/L NaF, 1% Triton X-100, 10 μL/mL protease inhibitor (Sigma-Aldrich), 10 μL/mL phosphatase I inhibitor (Sigma-Aldrich), 10 μL/mL phosphatase II inhibitor (Sigma-Aldrich), and 1.5 mg/mL benzamidine HCl. Tissue homogenates were centrifuged for 25 min at 15,000*g*, and supernatants were stored at −80°C. Solubilized proteins (30–40 μg) were run on a 4–20% SDS-PAGE (Bio-Rad, Hercules, CA), transferred onto nitrocellulose membrane (Hybond ECL; Amersham Biosciences), and blotted with the following primary antibodies: NPRA (Abcam), NPRC (Sigma-Aldrich), Akt, phospho-Akt

Ser473, p-Thr180/Tyr182-p38 MAPK, p38 MAPK, HSL, phospho-HSL Ser660, and phospho-HSL Ser565 (all Cell Signaling Technology, Beverly, MA). Subsequently, immunoreactive proteins were blotted with secondary horseradish peroxidase–coupled antibodies and revealed by enhanced chemiluminescence reagent (SuperSignal West Dura or SuperSignal West Femto; Thermo Scientific), visualized using the ChemiDoc MP Imaging System, and data analyzed using the Image Laboratory 4.1 version software (Bio-Rad). GAPDH (Cell Signaling Technology) and α-tubulin (Sigma-Aldrich) were used as internal controls for skeletal muscle and myotubes, and β-actin (Cell Signaling Technology) was used as internal control for adipose tissues.

Real-Time qRT-PCR

Total from tissues and primary myotubes were processed for RNA extraction using the RNeasy RNA mini kit (Qiagen GmbH, Hilden, Germany). After reverse transcription of total RNA (1 μg), samples were analyzed on a StepOnePLus real-time PCR system (Applied Biosystems). All primers were obtained from Applied Biosystems and presented in Supplementary Table 5. All expression data were normalized by the $2^{(\Delta Ct)}$ method using 18S as internal control.

Statistics

Statistical analyses were performed using GraphPad Prism 5.0 for Windows (GraphPad Software Inc.). Normal distribution and homogeneity of variance of the data were tested using Shapiro-Wilk and F tests, respectively. One-way ANOVA followed by Tukey post hoc tests and Student *t* tests were performed to determine differences between groups, interventions, and treatments. Two-way ANOVA followed by Bonferonni post hoc tests were applied when appropriate. Linear regression was performed after log transformation of nonparametric data. The false discovery rate for multiple testing was controlled by the Benjamini-Hochberg procedure with $P_{adj.}$ values ≤ 0.05 as threshold. All values in figures and tables are presented as mean ± SEM. Statistical significance was set at $P < 0.05$.

RESULTS

Muscle NPRA and NPRC Proteins Relate to Insulin Sensitivity in Humans

Muscle NPRA protein expression was investigated in human vastus lateralis biopsies of healthy volunteers with varying degrees of body fat and insulin sensitivity. We observed that muscle NPRA protein was inversely related to body fat (Fig. 1*A* and Supplementary Fig. 1*A*), BMI, fasting insulin, and indices of insulin resistance (Supplementary Table 2). In addition, muscle NPRA correlated positively with whole-body insulin sensitivity measured by euglycemic-hyperinsulinemic clamp (Fig. 1*B*) and the insulin sensitivity index (Supplementary Fig. 1*B*) and negatively with total muscle saturated ceramide content (Fig. 1*C*). Importantly, muscle NPRA protein content was significantly reduced (−65%) in obese subjects when compared with age-matched lean subjects (Fig. 1*D*). Conversely, muscle NPRA protein was upregulated (1.8-fold) together with insulin sensitivity (+37%, 5.4 ± 0.6 vs. 7.4 ± 1.1 mg/min/kg for pre- and postcalorie restriction, respectively, $P = 0.03$) in obese subjects with IGT in response to diet-induced weight loss (Fig. 1*E*). Finally, muscle NPRC protein content was unchanged in obese versus lean individuals with normal glucose tolerance (0.41 ± 0.08 vs. 0.29 ± 0.07 arbitrary units [A.U.], not significant) but increased significantly in obese individuals with IGT and T2D (Fig. 1*F*). The ratio of NPRA to NPRC protein was significantly reduced in obese versus lean subjects (2.1 ± 0.3 vs. 3.6 ± 0.2 A.U., respectively, $P = 0.0005$) and increased in obese subjects in response to calorie restriction (0.38 ± 0.16 vs. 0.14 ± 0.04 A.U., respectively, $P = 0.08$). Together this suggests that skeletal muscle NPR expression relates to insulin sensitivity in humans and is altered in obesity and T2D.

Impaired NPRA Expression in Skeletal Muscle and Fat of Diet-Induced Obese Mice

Since both skeletal muscle and adipose tissue are known as key target tissues of NP, both in humans and mice, we further examined NPR expression in metabolic tissues of chow-fed versus HFD-fed mice. In line with human data, we found a significant downregulation of NPRA protein in skeletal muscle (Fig. 2*A* and *D*), as well as in white (Fig. 2*B* and *D*) and brown fat (Fig. 2*C* and *D*) of HFD-fed mice. No significant change in NPRC protein content was found in skeletal muscle and brown fat, whereas NPRC protein decreased significantly in white fat (0.48 ± 0.08 vs. 0.14 ± 0.04 A.U. for chow and HFD, respectively, $P < 0.05$). Plasma BNP levels were unchanged in HFD-fed mice compared with chow-fed mice (Fig. 2*E*). Collectively, as in humans, our data indicate a reduced NPRA expression in skeletal muscle of obese mice.

Chronic BNP Infusion Protects Against HFD-Mediated Obesity and Glucose Intolerance

Since muscle NPRA is associated with insulin sensitivity in humans, we assessed the effect of acute and chronic BNP infusions on glucose tolerance and insulin sensitivity in chow-fed and HFD-fed mice. Acute intraperitoneal BNP injection did not affect fasting blood glucose levels over a time course of 30 min (Supplementary Fig. 2*A*) and had no effect on glucose excursion during an intraperitoneal GTT (Supplementary Fig. 2*B*). No effect of acute BNP injection was also seen on glucose disposal in skeletal muscle (Supplementary Fig. 2*C*). We further assessed the influence of acute NP treatment on basal and insulin-stimulated glucose uptake in human primary skeletal muscle cells. No effect of increasing doses of ANP and BNP on glucose uptake was observed (Supplementary Fig. 3). From these data we concluded that NPRA signaling does not acutely modulate glucose uptake in skeletal muscle.

Based on a previous study (32), we next infused BNP at a dose of 5 ng/kg/min, which raised plasma BNP levels by ∼40% (data not shown). BNP-treated mice had a similar body weight (Fig. 3*A*) and body composition (Fig. 3*B*) after

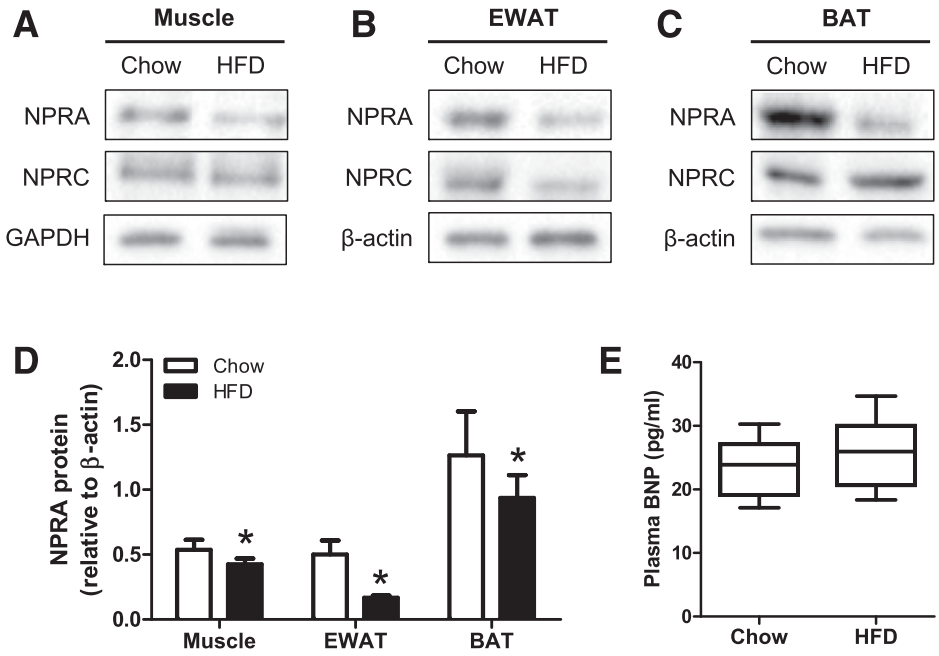

Figure 2—Defective NPR expression in metabolic tissues of diet-induced obese mice. Representative blots of NPRA and NPRC proteins in skeletal muscle (*A*), epididymal white adipose tissue (EWAT) (*B*), and brown adipose tissue (BAT) (*C*) of chow-fed and HFD-fed mice. Quantitative bar graph of NPRA protein (*D*) and overnight fasting plasma BNP (*E*) levels in chow and HFD-fed mice. *$P < 0.05$ vs. chow-fed mice (n = 8–10 per group).

saline and BNP treatment. Chronic BNP treatment significantly reduced fasting blood glucose levels in mice fed an HFD for 8 or 12 weeks (Fig. 3*C*). Lower blood glucose in the fasting state was also accompanied by improved glucose tolerance (Fig. 3*D*) despite no change in fasting and peak insulin at 15 min during the intraperitoneal GTT (Fig. 3*E*). In conclusion, whereas acute BNP treatment has no effect on insulin sensitivity, chronic BNP treatment improves glucose tolerance in HFD-fed mice.

Impaired NPR Signaling in Skeletal Muscle of Obese Diabetic Mice Contributes to the "NP Handicap"

We next examined NPR expression in metabolic tissues from leptin receptor–deficient mice (*db*/*db*) that become spontaneously obese and T2D by the age of 8 weeks. In line with data in human skeletal muscle and HFD-fed mice, NPRA protein was downregulated in white (Fig. 4*B–D*) and brown fat (Fig. 4*C* and *D*) of *db*/*db* mice compared with control *db*/+ mice. In agreement with data in individuals with IGT/T2D (Fig. 1*E*), we noted a remarkable upregulation of NPRC in skeletal muscle (Fig. 4*A–E*), as well as in white (Fig. 4*B–E*) and brown (Fig. 4*C–E*) fat of *db*/*db* mice. Overall the NPRA-to-NPRC protein ratio was markedly downregulated in muscle and fat of *db*/*db* mice (Fig. 4*F*) and was associated with dramatically lower levels of plasma BNP in *db*/*db* mice (−80%, $P < 0.05$) (Fig. 4*G*). This was also associated with a remarkable downregulation of p38 MAPK phosphorylation in skeletal muscle of *db*/*db* mice (−55%, $P < 0.001$) (Supplementary Fig. 4). Importantly, muscle NPRC was negatively correlated with plasma BNP levels (Supplementary Table 3). These changes in NPR signaling and plasma NP characterized an "NP handicap" of *db*/*db* mice. No association was found between white and brown fat NPRC protein and plasma BNP levels (data not shown). However, muscle NPRC was positively related to fasting blood glucose, insulin, and HbA_{1c} (Supplementary Table 4), again suggesting a link between defective skeletal muscle NPR signaling and impaired glucose control. Collectively, these data suggest that obesity and T2D are accompanied by profound changes in NPR expression and signaling in skeletal muscle, which may contribute to reduced plasma BNP levels.

Chronic BNP Infusion Improves Blood Glucose Control in Obese Diabetic Mice

We next studied the influence of chronic (4 weeks) BNP infusion on blood glucose control in *db*/*db* mice. BNP was infused at a dose of 10 ng/kg/min to induce a nearly twofold increase in plasma BNP levels with the goal of rescuing the "NP handicap." Despite no change in body weight (Fig. 5*A*) and composition (Fig. 5*B*), BNP-treated *db*/*db* mice displayed significantly improved blood glucose control, with reduced fasting plasma glucose (−21%) (Fig. 5*C*) and HbA_{1c} (−17%) (Fig. 5*D*). This improved blood glucose control occurred in the absence of noticeable changes in fasting insulin (Fig. 5*E*). In addition, insulin tolerance (Fig. 5*F*) and insulin responsiveness (area above the curve during the ITT, +36%, $P = 0.08$) were improved in BNP-treated mice. In summary, chronic BNP treatment improves blood glucose control and peripheral insulin sensitivity in obese diabetic mice independently of changes in body weight, thus suggesting a direct effect of NP on metabolic organs.

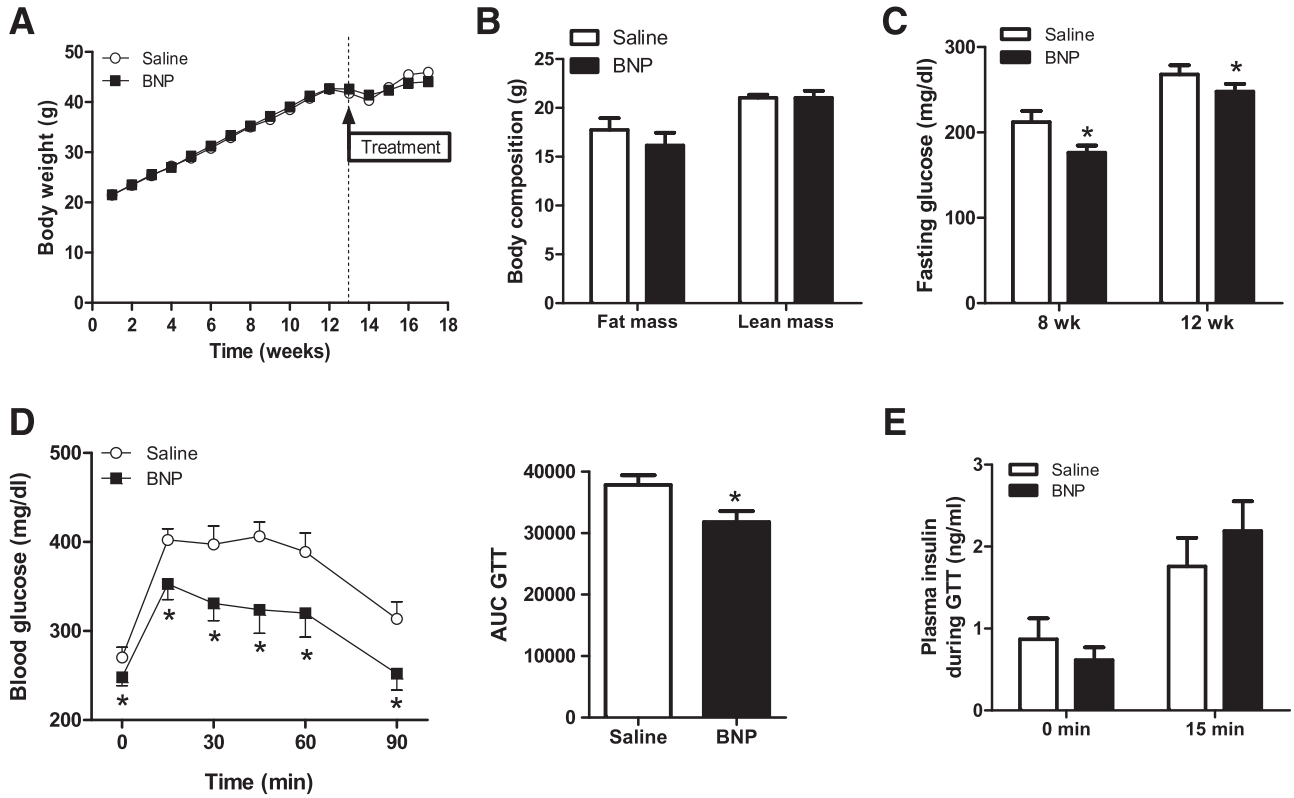

Figure 3—Chronic BNP infusion protects from HFD-induced obesity and glucose intolerance. C57BL/6J mice were treated for 4 weeks with saline (0.9% NaCl) or with BNP (5 ng/kg/min) via mini-osmotic pumps after 12 weeks of HFD. *A*: Follow-up of body weight during HFD and after mini-pump was placed. *B*: Body composition at the end of treatment in saline- and BNP-treated obese mice. *C*: Overnight fasting blood glucose in BNP-treated mice after 8 and 12 weeks of HFD. *D*: Time course of blood glucose levels during an intraperitoneal GTT and corresponding area under the curve (AUC). *E*: Plasma insulin after a 6-h fast (0 min) and 15 min after glucose bolus injection. *$P < 0.05$ vs. saline (n = 8–10).

Enhanced Insulin Signaling, Reduced Lipotoxicity, and Increased Lipid Oxidative Capacity in Skeletal Muscle of BNP-Treated Mice

We next studied the mechanism by which chronic BNP treatment improved blood glucose control and muscle insulin sensitivity in both HFD-fed and *db/db* mice. Insulin sensitivity is inhibited by the accumulation of toxic lipids such as DAGs and ceramides in skeletal muscle and liver (33,34). No significant change in total DAG and ceramides was found in the liver of BNP-treated *db/db* (Supplementary Fig. 5*A* and *B*) and HFD-fed mice (Supplementary Fig. 6*A* and *B*). No change as well in mRNA levels of genes involved in fat oxidation and glucose metabolism was observed after BNP infusion in the liver of *db/db* (Supplementary Fig. 5*C*) and HFD-fed mice (Supplementary Fig. 6*C*). Similarly, no change in the expression level of classical thermogenic genes in brown and white fat depots was observed in BNP-treated *db/db* (Supplementary Fig. 7) and HFD-fed mice (Supplementary Fig. 8). No change in *Ucp1* mRNA levels was noted as well in inguinal white adipose tissue (data not shown). However, we observed a muscle-autonomous improvement of insulin-mediated Akt (46%, P = 0.02) and p38MAPK phosphorylation (278%, P = 0.06) (Fig. 6*A*), which was paralleled by a reduced content of total and main species (data not shown) of ceramides (−17%) (Fig. 6*B*) as well as total and main species (data not shown) of sphingomyelin (−19%) (Fig. 6*C*) in skeletal muscle of BNP-treated HFD-fed mice, as well as a reduced content of total ceramides in *db/db* mice (52.4 ± 4.4 vs. 40.0 ± 5.1 ng/μg protein for *db/+* and *db/db* mice, respectively, $P < 0.05$). The content of total and subspecies of DAGs was also reduced in BNP-treated HFD-fed mice (ANOVA $P < 0.05$) (Fig. 6*D*). This lower lipotoxic pressure was paralleled by an upregulation of muscle palmitate oxidation rate (+46%) (Fig. 6*E*) and of *PGC1α* mRNA levels in HFD-fed mice (Fig. 6*F*) and in *db/db* mice (+32%, P = 0.08). Collectively, the data indicate that chronic BNP treatment improves insulin sensitivity in skeletal muscle by reducing lipotoxicity and upregulating fat oxidative capacity in a PGC1α-dependent manner in obese and diabetic mice.

Chronic NP Treatment Reduces Lipotoxicity and Enhances Lipid Oxidative Capacity in Human Primary Myotubes

We previously demonstrated a functional NPR signaling in human primary myotubes (20). Because NP are known to activate lipolysis in human adipocytes (35,36), we

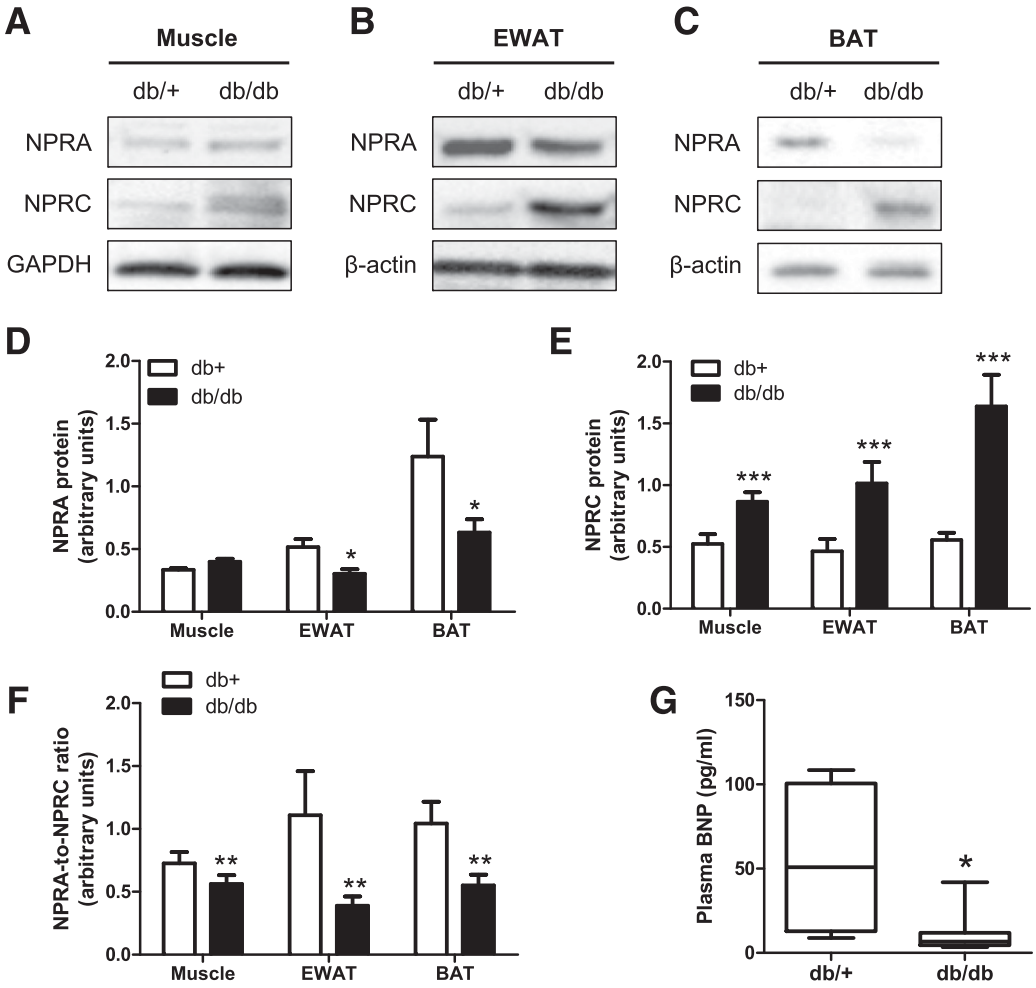

Figure 4—Defective NPR signaling in metabolic tissues of obese diabetic mice. Representative blots of NPRA and NPRC proteins in skeletal muscle (*A*), epididymal white adipose tissue (EWAT) (*B*), and brown adipose tissue (BAT) (*C*) of *db*/*db* and *db*/+ mice. Quantitative bar graph of NPRA (*D*), NPRC protein (*E*), NPRA-to-NPRC protein ratio (*F*), and overnight fasting plasma BNP levels in *db*/*db* and *db*/+ mice (*G*). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$ vs. *db*/+ mice (n = 8–10 per group).

studied here the acute effect of NP treatment on lipid metabolism. Acute treatment of myotubes with BNP did not influence lipid storage, endogenous TAG-derived fatty acid (FA) release (i.e., lipolysis) (Supplementary Fig. 9*A*), and endogenous TAG-derived FA oxidation (Supplementary Fig. 9*B*). We further tested whether NP could activate one of the rate-limiting enzymes of lipolysis. Acute BNP treatment of human myotubes did not influence hormone-sensitive lipase phosphorylation either on the activating Ser660 residue (Supplementary Fig. 9*C*) or on the inhibitory Ser565 residue (Supplementary Fig. 9*D*). In contrast, chronic treatment with NP for 3 days robustly reduced total lipid accumulation and total TAG and DAG content (one-way ANOVA $P < 0.001$) (Fig. 7*A*–*C*). In line with ex vivo muscle data in mice (Fig. 6), reduced lipid accumulation was concomitant with an upregulation of palmitate oxidation rate (+27 and +19%, respectively, for ANP and BNP treatment) (Fig. 7*D*), and a significant induction of *PGC1α* gene expression (Fig. 7*E*), which was independent of PPARδ activation (Fig. 7*F*). No change in myogenic gene expression and differentiation of myoblasts into myotubes was observed in response to chronic NP treatment (data not shown). Based on the findings that muscle NPRA protein relates inversely to saturated ceramide content in human skeletal muscle (Fig. 1*C*) and that chronic BNP treatment reduces ceramide content in skeletal muscle of HFD-fed mice (Fig. 6*B*), we assessed the influence of chronic NP treatment on ceramide content in human primary myotubes. No significant effect of NP treatment on the content of total ceramides and various ceramide species (Supplementary Fig. 10) was noticed in the basal condition with FA-free BSA treatment. When myotubes were challenged overnight with 500 μmol/L of palmitate/BSA to induce ceramide production (2.7-fold, $P = 0.001$), we observed a significant decrease of ~30% in total and various ceramide species analyzed in response to chronic ANP and BNP treatment (Fig. 7*G*). In summary, chronic NP treatment protects against lipotoxicity by upregulating lipid oxidative capacity in human primary myotubes.

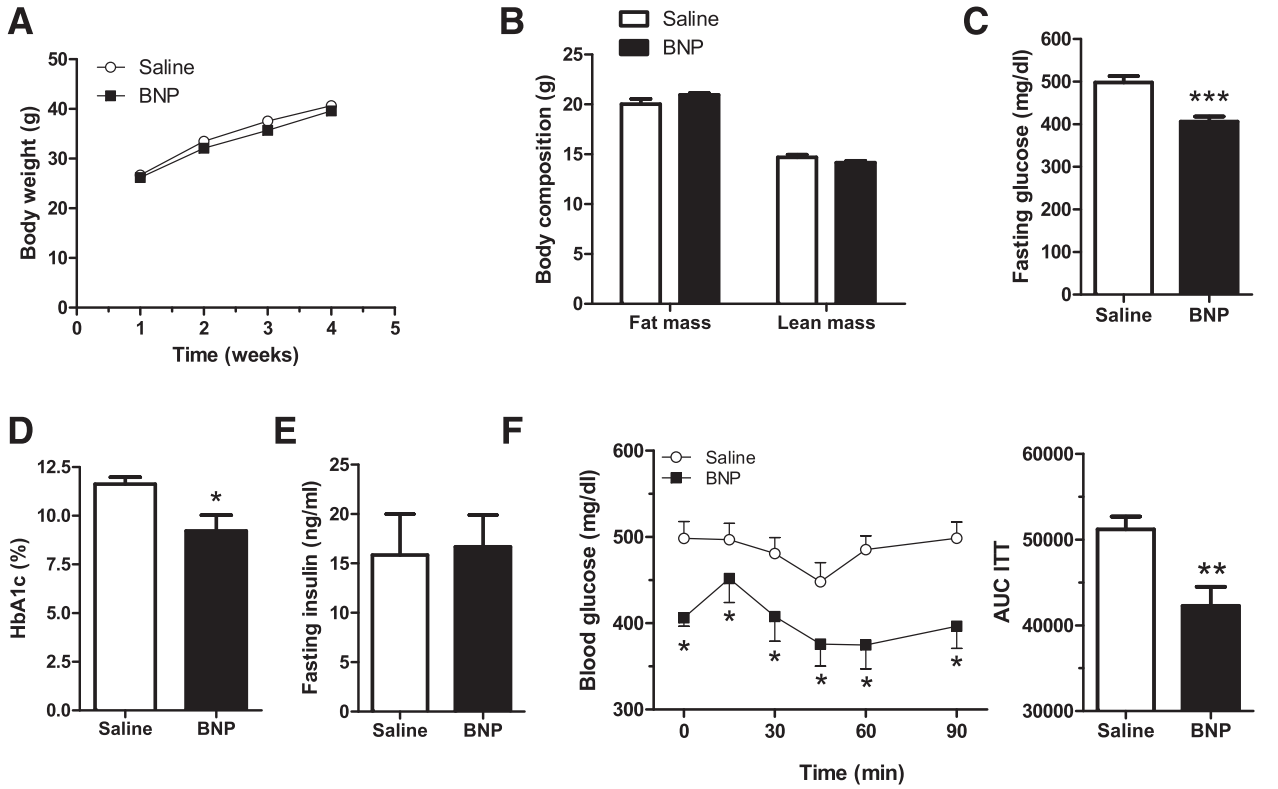

Figure 5—Chronic BNP infusion improves blood glucose control in obese diabetic mice. Five-week-old *db/db* mice were chronically treated for 4 weeks with saline (0.9% NaCl) or with BNP (10 ng/kg/min) via mini-osmotic pump. *A*: Follow-up of body weight over 4 weeks of treatment with saline or BNP. *B*: Body composition at the end of treatment. Overnight fasting blood glucose (*C*), HbA_{1c} (*D*), and overnight fasting insulin (*E*) were measured after 4 weeks of BNP treatment. *F*: Time course of blood glucose levels during an intraperitoneal ITT and corresponding area under the curve (AUC) after 4 weeks of treatment. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. saline-treated *db/db* mice (n = 8–10).

DISCUSSION

Although longitudinal prospective studies indicated that high baseline levels of plasma NP confer a reduced risk of developing T2D (5,6), no study so far had demonstrated a mechanistic link between NP biological activity and T2D. We believe our data provide the first evidence that NPRA signaling in skeletal muscle is necessary for the maintenance of long-term insulin sensitivity by regulating lipid oxidative capacity and metabolism (Fig. 8). Our data show for the first time that muscle NPRA signaling is impaired in the context of obesity and glucose intolerance in humans and mice. We also provide evidence that upregulation of NPRC in muscle tissue can contribute to the "NP handicap" observed in T2D. Last but not least, increasing NP levels in obese and diabetic mice, with the goal of rescuing the "NP handicap" and so a normal NPRA signaling tissue response, markedly improves blood glucose control and insulin sensitivity in skeletal muscle.

We first observed a significant positive association between muscle NPRA protein and insulin sensitivity measured by clamp in humans, at a dose that mainly reflects skeletal muscle insulin sensitivity. This observation is consistent with the negative association that we found between muscle NPRA and body fat, and between muscle NPRA and muscle total saturated ceramide content, two factors negatively influencing whole-body and muscle insulin sensitivity (33,34). To our knowledge, this is the first study reporting an association between skeletal muscle NPRA signaling and insulin sensitivity. This indicates that besides plasma NP levels, NPR signaling in skeletal muscle may influence insulin sensitivity. Additionally, muscle NPRA protein was dramatically downregulated in obese individuals while increased in response to diet-induced weight loss and related improvement in insulin sensitivity. Although the biological factors modulating muscle NPRA protein content were not investigated in the current study, the data suggest that muscle NPRA behaves as a determinant of insulin sensitivity. Moreover, upregulation of muscle NPRC as glucose tolerance deteriorates in obese subjects with IGT and T2D can further repress biological activation of muscle NPRA and contribute to the "NP handicap" in the long-term. Considering that muscle mass represents up to 40% of total body weight, even a moderate increase in muscle NPRC expression could largely reduce plasma NP levels by an increased rate of clearance. Muscle NPRC might be induced by high blood insulin levels in obese subjects as

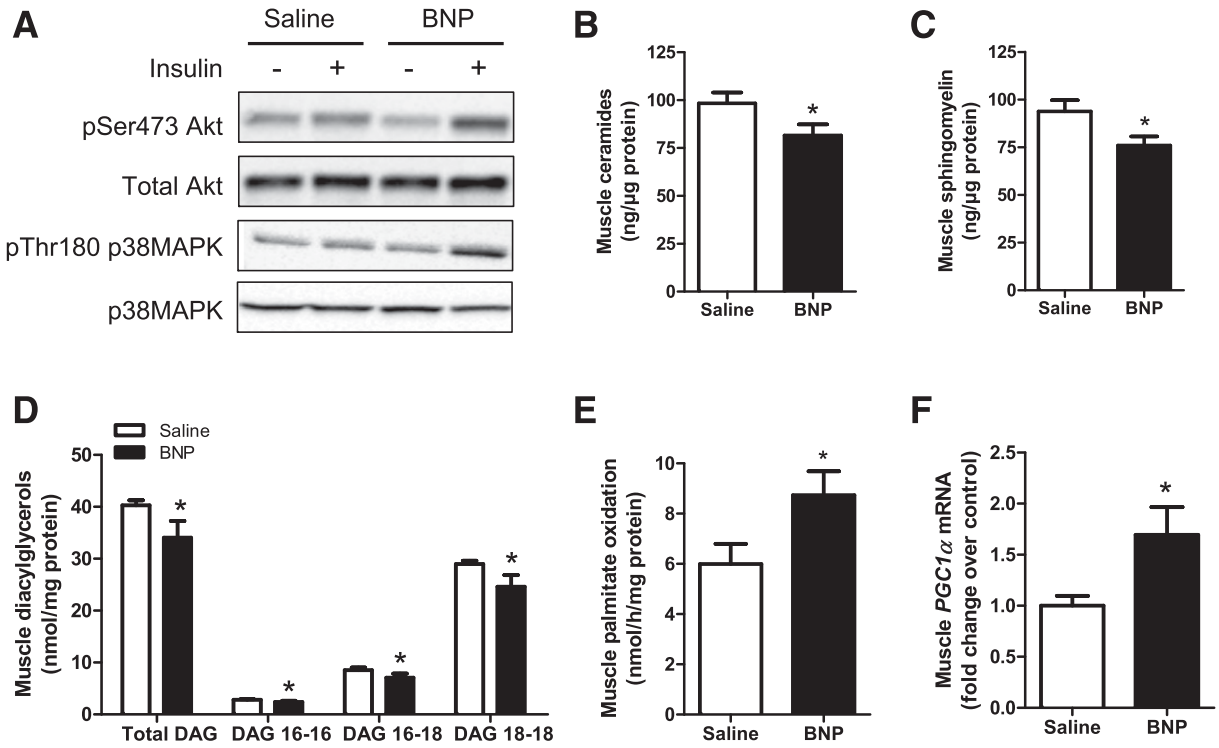

Figure 6—Muscle-autonomous improvement of insulin signaling and reduced lipotoxicity in skeletal muscle of BNP-treated obese mice. *A*: Extensor digitorum longus muscles were incubated ex vivo in absence (−) or presence of 100 nmol/L of insulin (+), and phosphorylated and total Akt and p38 MAPK were measured by Western blot. Total ceramides (*B*), total sphingomyelin (*C*), total and DAG subspecies content (*D*), ex vivo palmitate oxidation rate (*E*), and *PGC1α* gene expression (*F*) in skeletal muscle of HFD-fed mice treated with saline and BNP 5 ng/kg/min. *$P < 0.05$ vs. saline (n = 8–10).

glucose tolerance worsens independently of blood glucose concentrations as previously shown in adipose tissue (37). Although obese control and IGT/T2D were not age matched, increased expression of NPRC in skeletal muscle appeared independent of age since no correlation between age and muscle NPRC protein was found. Importantly, these findings in human muscle were largely replicated in obese diabetic mice. NPRC protein content was increased in skeletal muscle, white fat, and brown fat of obese diabetic mice, but only muscle NPRC protein negatively correlated with plasma BNP levels, reflecting that an increased plasma BNP clearance by the muscle can contribute to the "NP handicap" observed in these mice. Our data are in line with other studies demonstrating that elevated *NPRC* mRNA levels in white fat relate to metabolic dysfunction in mice and humans (22,38,39). Our data also provide a mechanistic understanding of the tight link observed between the NP handicap and insulin resistance independently of obesity in humans (40). The "NP handicap" concept is supported by the fact that the half-life of NP in the blood circulation is substantially increased in NPRC knockout mice and the biological activity of NP significantly increased in target tissues (41). Importantly, the altered NPRA-to-NPRC protein ratio in skeletal muscle was accompanied by a marked alteration of p38 MAPK phosphorylation in *db*/*db* vs. *db*/+ mice, thus indicating a potential signaling defect, considering that p38 MAPK is recognized as a canonical downstream molecular effector of the NPR signaling pathway (19).

Despite the observed link between muscle NPRA and insulin sensitivity, acute injection of BNP had no impact on fasting blood glucose, glucose tolerance, and muscle insulin sensitivity in mice. Furthermore, no acute effect of NP on glucose uptake was observed in human primary myotubes. These findings are in agreement with at least one human study reporting no acute effect of BNP on insulin sensitivity and insulin secretion (42). Altogether these data indicate that NP signaling does not acutely modulate skeletal muscle glucose uptake in vivo. We therefore performed chronic BNP infusion studies in HFD-fed and obese diabetic *db*/*db* mice to assess the long-term influence of BNP treatment on blood glucose control and insulin sensitivity. BNP was preferred for infusion studies as it has a higher half-life than ANP (14). Strikingly, chronic BNP infusion, at doses mimicking a physiological increase of the peptide and targeted to rescue the "BNP handicap" and/or a normal tissue NPRA signaling response, very significantly improved blood glucose control in both mouse models of obesity-induced glucose intolerance and T2D. We observed >20% reduction in fasting blood glucose levels as well as >15% decrease in HbA_{1c}, which is clinically meaningful and

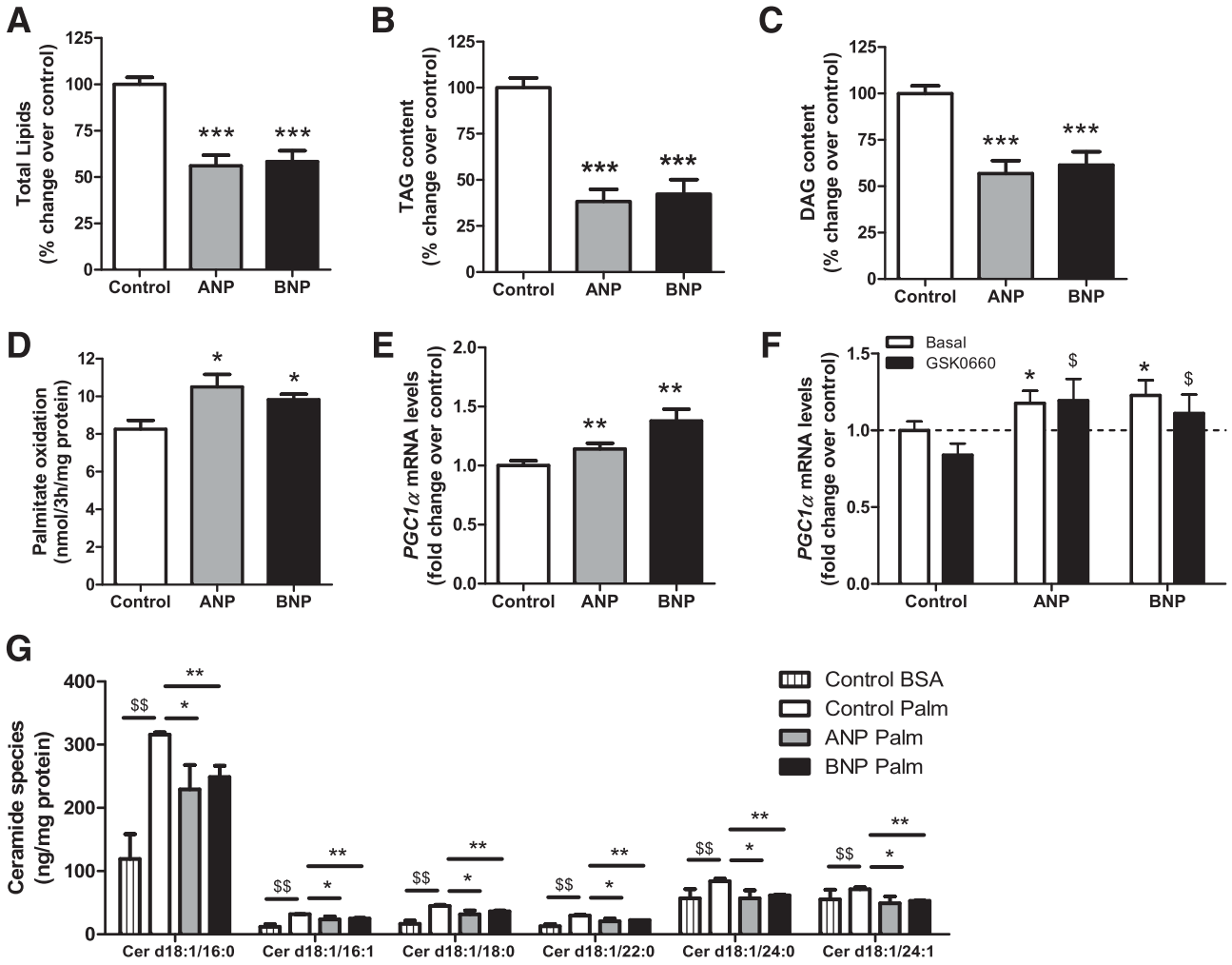

Figure 7—Chronic NP treatment reduces lipotoxicity and increases lipid oxidative capacity in human primary myotubes. Total lipid accumulation (*A*), TAG (*B*), and DAG (*C*) content were determined with [1-^{14}C]oleate after 3-day chronic treatment with 100 nmol/L of ANP and BNP in human differentiated myotubes. *D*: Total palmitate oxidation rate was also measured in response to chronic ANP and BNP treatment. *PGC1α* gene expression in response to 3-day treatment with ANP and BNP (*E*) and in presence or absence of 500 nmol/L of the selective PPARδ antagonist GSK0660 (*F*). **P* <0.05, ***P* < 0.01, ****P* < 0.0001 vs. control (*A–E*); **P* < 0.05 vs. basal; $*P* < 0.05 vs. GSK0660 (*n* = 4–10). *G*: Ceramide species content in human primary myotubes in basal condition (BSA), and in response to overnight treatment with 500 μmol/L of palmitate/BSA (Palm) in control myotubes and in response to 3-day treatment with ANP or BNP. $$*P* < 0.01 vs. control BSA; **P* < 0.05; ***P* < 0.01 vs. control palm (*n* = 4).

strongly reduces the risk of T2D complications (43). Reduced blood glucose levels during fasting and upon oral glucose challenge occurred in the absence of changes in blood insulin levels, indicating an improved metabolic clearance of glucose and insulin sensitivity. These findings are in agreement with other studies showing that increasing plasma BNP levels either pharmacologically (32) and/or genetically (22) improves glucose tolerance in obese mice. Preliminary evidence from our laboratory indicates that ANP-knockout mice are insulin resistant under normal chow diet compared with their wild-type littermates (data not shown), again arguing for a direct physiological link between NP signaling and insulin sensitivity.

Improved blood glucose control and insulin sensitivity were independent of significant changes in total DAGs and ceramides in liver, neither with noticeable changes in expression level of key metabolic genes in liver and thermogenic genes in white and brown fat of BNP-treated *db/db* and HFD-fed mice. However BNP-treated obese mice had an increased insulin-mediated Akt activation in skeletal muscle. Because Akt activation and phosphorylation are inhibited by lipotoxic lipids such as ceramides and DAGs (33,34), we measured ceramides and DAGs in skeletal muscle. In agreement with the negative correlation found in humans between muscle NPRA and ceramide content, we found a reduced level of total and main species of ceramides as well as main species of sphingomyelin in muscle of both HFD-fed and *db/db* mice chronically treated with BNP. Ceramides inhibit Akt activation and are produced de novo from saturated FAs and from sphingomyelin degradation (33). We also observed reduced

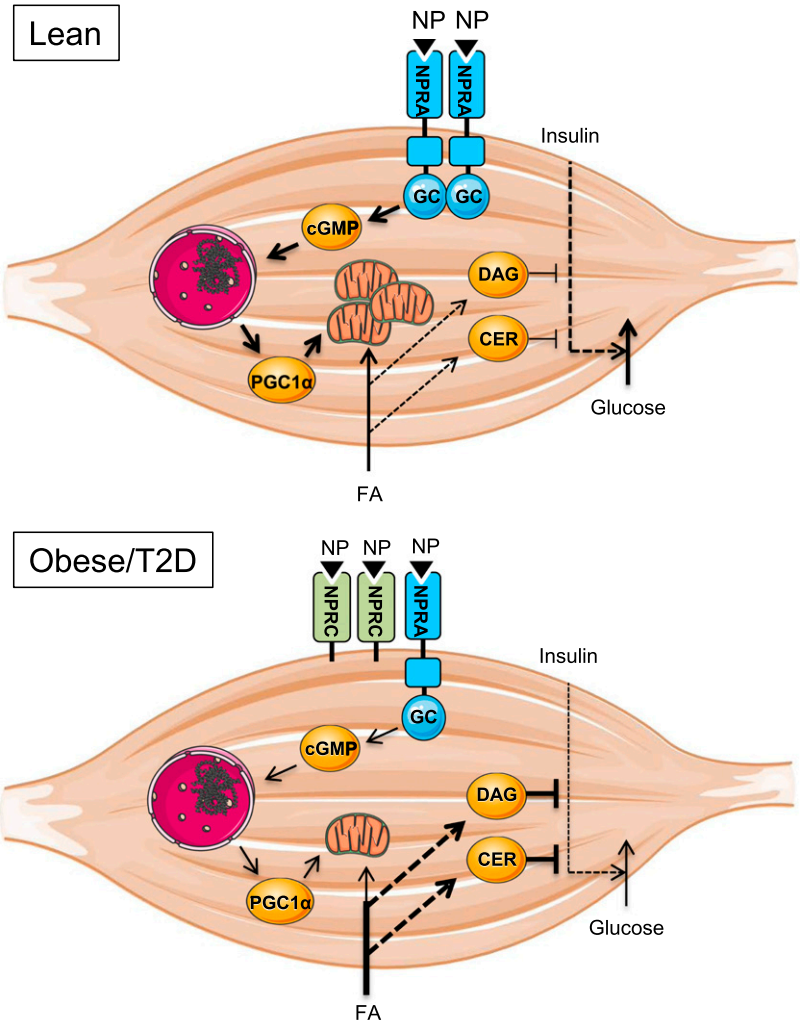

Figure 8—Model linking natriuretic peptide signaling in skeletal muscle and insulin sensitivity. In lean individuals, NPRA activation (bearing an intrinsic guanylyl cyclase activity [GC]) by circulating NP induces PGC1α expression in a cGMP-dependent manner that leads to increased fat oxidation rates and low levels of lipotoxic DAGs and ceramides (CER), which maintain a normal insulin responsiveness in skeletal muscle. Defective NPR signaling in skeletal muscle during obesity contributes to reduced fat oxidative capacity, increased lipotoxicity, and insulin resistance. Upregulation of NPRC in skeletal muscle as glucose tolerance deteriorates with obesity further inhibits the biological activation of NPRA by circulating NP and reduces NP circulating levels. Solid arrow line, direct effect; dashed arrow line, indirect effect.

muscle total DAG levels in BNP-treated mice. Interestingly, the reduced muscle lipotoxic lipid level was accompanied by a significant upregulation of muscle fat oxidative capacity and *PGC1α* gene expression. To demonstrate that elevated lipid oxidative capacity can reduce lipid accumulation, we chronically treated human primary myotubes with NP and showed increased palmitate oxidation rates and robustly reduced total lipid, TAG, and DAG accumulation. Chronic NP treatment also prevented palmitate-induced ceramide production in human primary myotubes. Although the precise mechanism was not investigated, it is likely that NP treatment reduces de novo ceramide production by increasing palmitate oxidation. We also show that NP-mediated elevated lipid oxidation involved the induction of PGC1α, which was independent of PPARδ activation. We and others previously described a cGMP-dependent induction of PGC1α gene expression by NP in white fat and skeletal muscle cells (19,20). PPARδ can be activated by lipid ligands derived from endogenous TAG lipolysis (31,44). In contrast to what has been shown in human fat cells (35,36), acute NP treatment of human primary myotubes did not influence the rate of lipolysis and TAG-derived FA oxidation or HSL phosphorylation at key regulatory sites.

Our findings provide the first evidence that NPRA signaling in skeletal muscle is pivotal for the maintenance of long-term insulin sensitivity by regulating lipid oxidative capacity through a PGC1α-dependent pathway. We also provide strong evidence that NPR signaling in skeletal muscle relates to insulin sensitivity and is disrupted in humans and mice with obesity and diabetes. Increasing plasma BNP

levels in obese diabetic mice remarkably improves blood glucose control and could prove a novel therapeutic avenue to alleviate obesity-related insulin resistance.

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SUPPLEMENTARY DATA

Supplementary Information for:

Defective natriuretic peptide receptor signaling in skeletal muscle links obesity to type 2 diabetes

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Inventory of Supplemental Information:

1. Supplemental Tables : 5
2. Supplemental Figures : 10

SUPPLEMENTARY DATA

Supplementary Table 1. Clinical characteristics of the subjects.

	Lean	**Obese**	**IGT/T2D**
Sex (male/female)	6/3	5/4	6/4
Age (yrs)	23.8±0.8	23.7±0.8	46.7±3.4[c,e]
Body weight (kg)	68.3±3.3	87.7±6.3[b]	103.3±4.1[c,d]
BMI (kg/m^2)	22.5±0.5	32.9±0.5[c]	34.3±1.2[c]
Body fat (%)	19.7±2.5	27.9±2.1[a]	30.1±1.4[c]
GDR (mg.min^{-1}.kg^{-1} FFM)	9.4±0.7	7.0±0.4[c]	5.5±0.5[c,d]

Data are Mean ± SEM. BMI: body mass index; GDR: glucose disposal rate; FFM: fat-free mass. [a]p<0.05, [b]p<0.01, [c]p<0.001 versus lean; [d]p<0.05, [e]p<0.01 versus obese.

SUPPLEMENTARY DATA

Supplementary Table 2. Correlation between muscle NPRA protein expression and biological variables in humans.

	Human muscle NPRA		
Variables	**r**	**p value**	**p adj. value**
Body weight (kg)	-0.46	0.06	0.06
BMI	-0.46	0.05	0.06
HOMA-IR	-0.48	0.04	0.06
rQUICKI	0.63	0.005	0.025
Fasting Insulin	-0.52	0.03	0.07

BMI: body mass index; HOMA-IR: homeostasis model assessment of insulin resistance; revised QUICKI. r: Spearman correlation coefficients; non adjusted p value; $p_{adj.}$ value: Benjamini-Hochberg false discovery rate considered statistically significant if ≤ 5%.

SUPPLEMENTARY DATA

Supplementary Table 3. Correlation between log fasting plasma BNP and biological variables in db/+ and db/db mice.

	Mouse Log [BNP]		
Variables	**r**	**p value**	**p adj. value**
Body weight (g)	-0.69	0.0007	0.0008
Fat Mass (%)	-0.79	< 0.0001	0.0002
Fasting glucose	-0.76	< 0.0001	0.0002
Fasting insulin	-0.85	< 0.0001	0.0007
HbA1c	-0.70	0.0006	0.0008
Fructosamines	-0.82	< 0.0001	0.0003
Muscle NPRC	-0.59	0.02	0.02

r: Spearman correlation coefficients; non adjusted p value; $p_{adj.}$ value: Benjamini-Hochberg false discovery rate considered statistically significant if ≤ 5%.

SUPPLEMENTARY DATA

Supplementary Table 4. Correlation between muscle NPRC protein expression and biological variables in db/+ and db/db mice.

	Mouse muscle NPRC		
Variables	**r**	**p value**	**p adj. value**
Fasting glucose	0.64	0.011	0.014
Fasting insulin	0.61	0.016	0.016
HbA1c	0.65	0.009	0.036
Fructosamines	0.65	0.012	0.024

HOMA-IR: homeostasis model assessment of insulin resistance. r: Spearman correlation coefficients; non adjusted p value; $p_{adj.}$ value: Benjamini-Hochberg false discovery rate considered statistically significant if ≤ 5%.

SUPPLEMENTARY DATA

Supplementary Table 5. List of mouse primer and probe sequences used for real-time qPCR

Taqman chemistry:

Gene symbol	Taqman Probe ID
UCP1	Mm01244861_m1
TFAM	Mm00447485_m1
GLUT1	Mm00441480_m1
GLUT2	Mm00446229_m1
GLUT4	Mm00436615_m1
CPT1β	Mm00487200_m1
GYS1	Mm00472712_m1
PCK1	Mm00440636_m1
18S	Hs99999901_s1

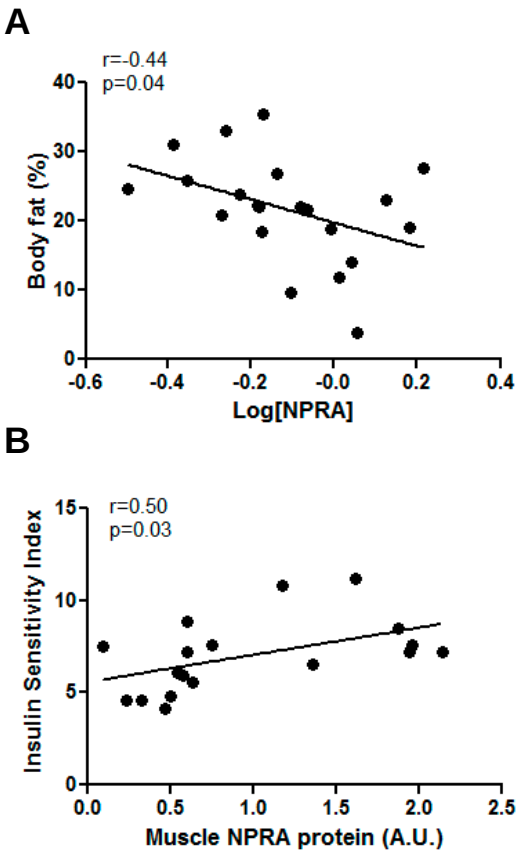
SYBR chemistry:

Gene symbol	Forward	Reverse
PPARα	AGTTCACGCATGTGAAGGCTG	TGTTCCGGTTCTTCTTCTGAATC
G6P	ACACCGACTACTACAGCAACAG	CCTCGAAAGATAGCAAGAGTAG
PGC1α	CTGTGTCACCACCCAAATCCTTAT	TGTGTCGAGAAAAGGACCTTGA

SUPPLEMENTARY DATA

Supplementary Figure S1. Correlation between muscle NPRA protein and clinical variables in humans

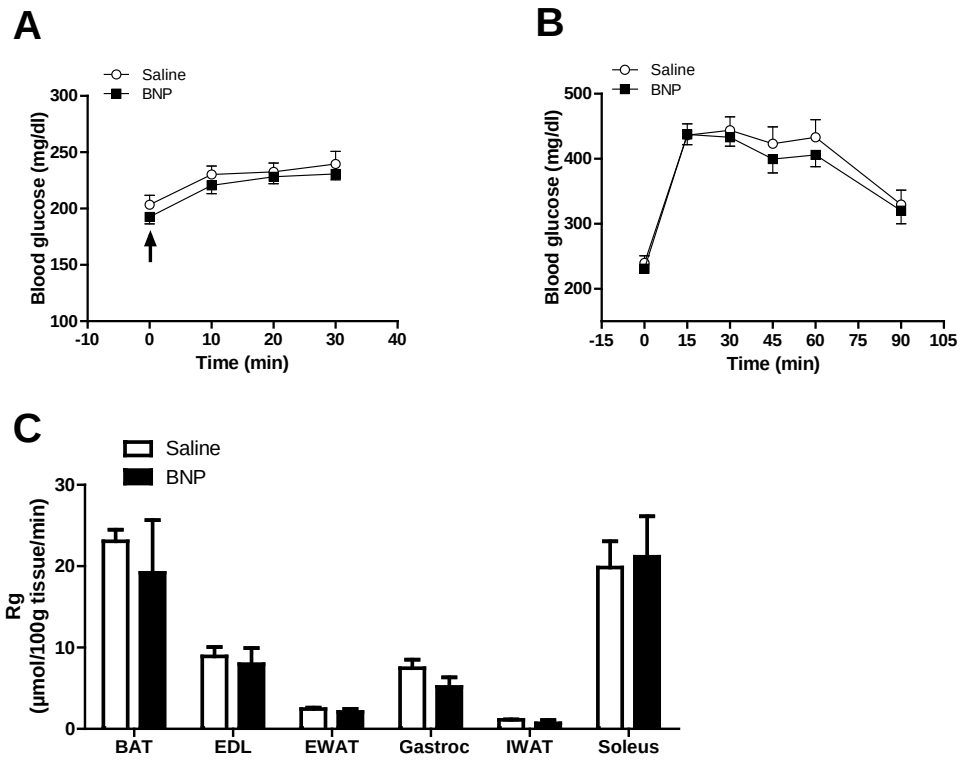
Correlation between *vastus lateralis* NPRA protein expression, and (**A**) percent body fat (n=21), and (**B**) the McAuley insulin sensitivity index measured during an oral glucose tolerance test in human subjects with normal glucose tolerance (n=18).



SUPPLEMENTARY DATA

Supplementary Figure S2. Acute effect of BNP injection on insulin sensitivity

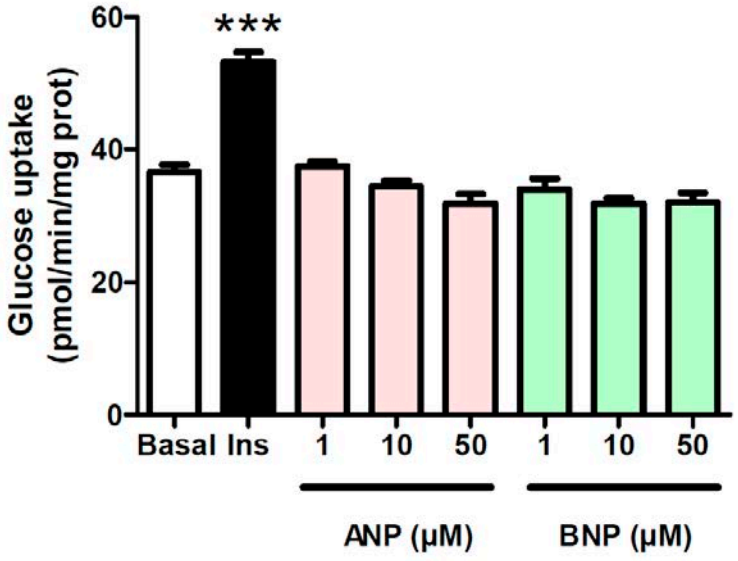
Fasted C57BL/6 mice fed standard chow diet were injected intraperitoneously with saline (0.9% NaCl) or with BNP (1 µg/kg) solution (arrow) and fasting blood glucose was measured (**A**) every 10 min in the basal state and (**B**) every 15 min during an i.p. GTT (n=11). (**C**) Tissue-specific glucose uptake was measured during a radiolabeled GTT with [2-^{3}H]deoxyglucose (n=6).



SUPPLEMENTARY DATA

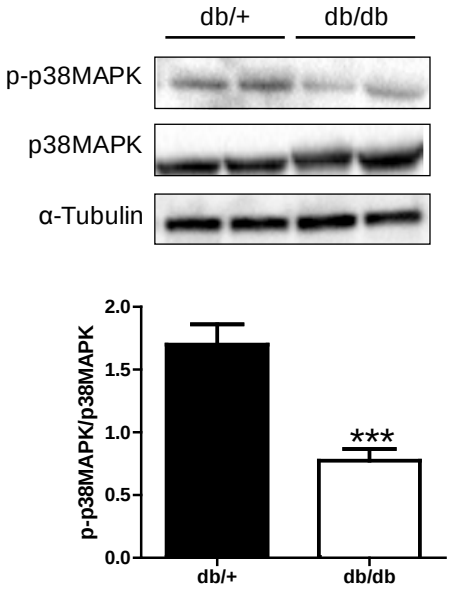
Supplementary Figure S3. Effect of acute NP treatment on glucose uptake in human primary myotubes

Glucose uptake was measured in presence of 1, 10 and 50 µM of ANP or BNP, and 1 µM of insulin in human primary myotubes. *** p<0.001 vs. saline (n=6).



SUPPLEMENTARY DATA

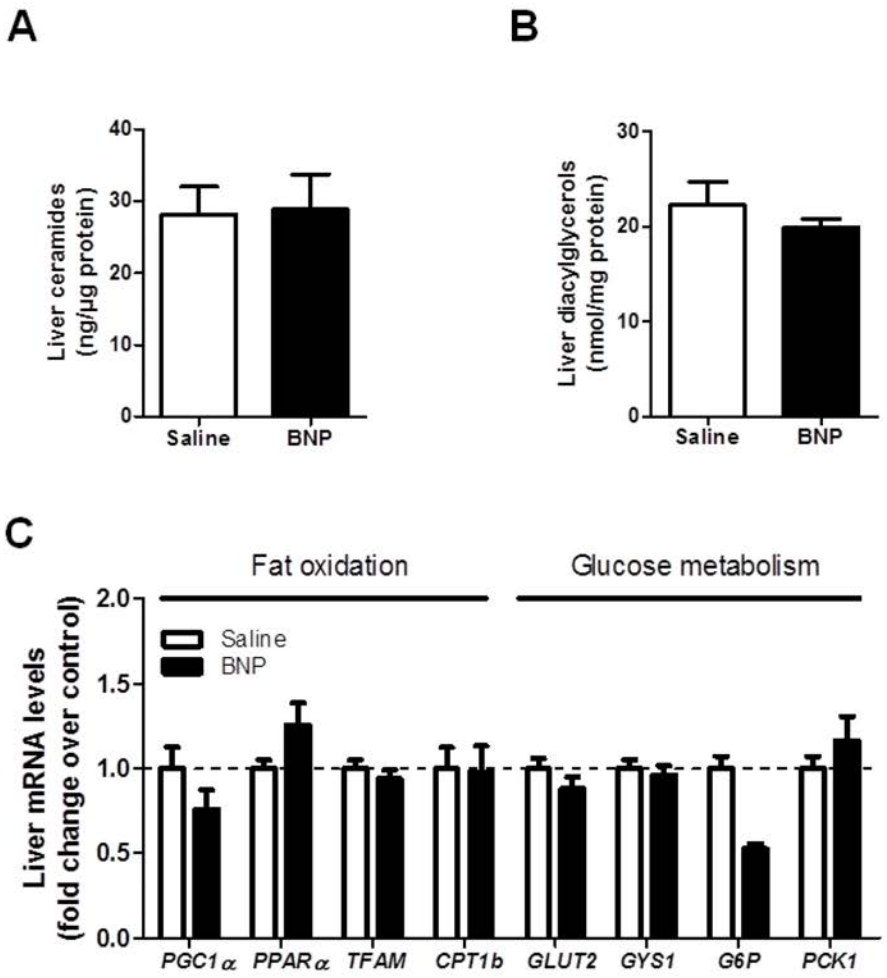
Supplementary Figure S4. Representative blots and quantitative bar graph of p38MAPK phosphorylation relative to total p38MAPK and α-tubulin in *gastrocnemius* muscle of db/+ versus db/db mice. ***p<0.0001 vs. db/+ (n=8).



SUPPLEMENTARY DATA

Supplementary Figure S5. Chronic BNP treatment does not change lipid levels and gene expression in liver of db/db mice

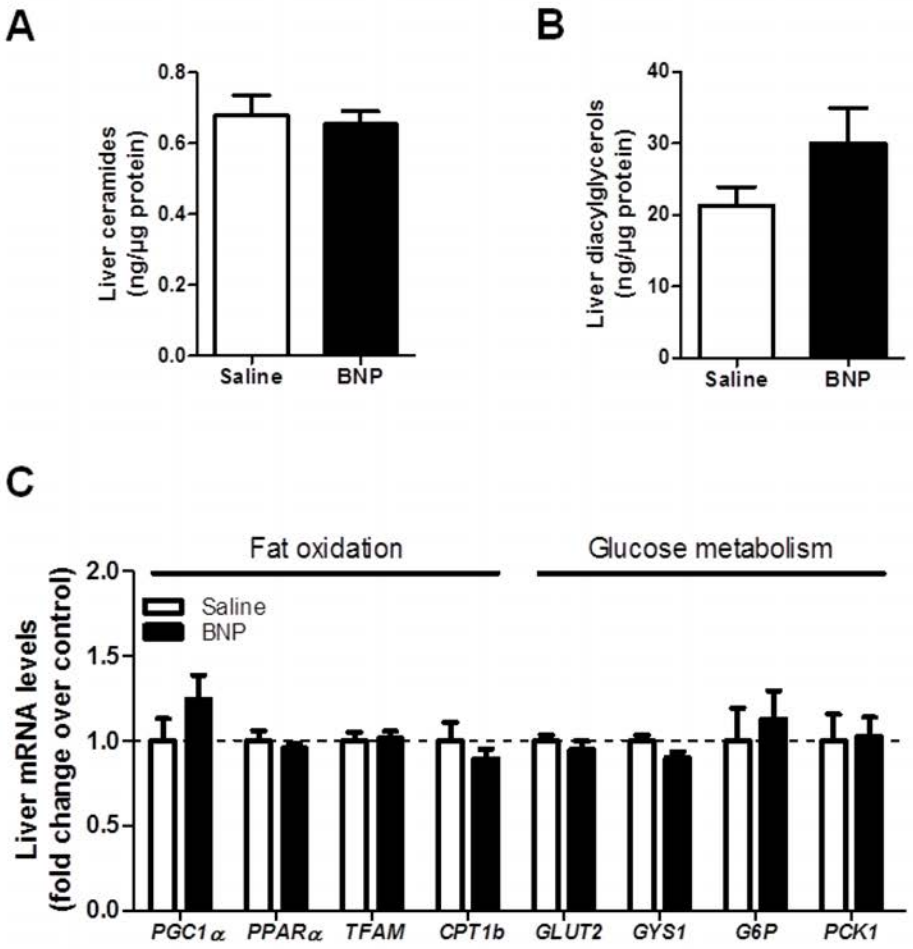
(**A**) Total ceramides, (**B**) total diacylglycerols levels, and (**C**) mRNA levels of genes involved in fat oxidation and glucose metabolism in liver of saline- and BNP-treated db/db mice.



SUPPLEMENTARY DATA

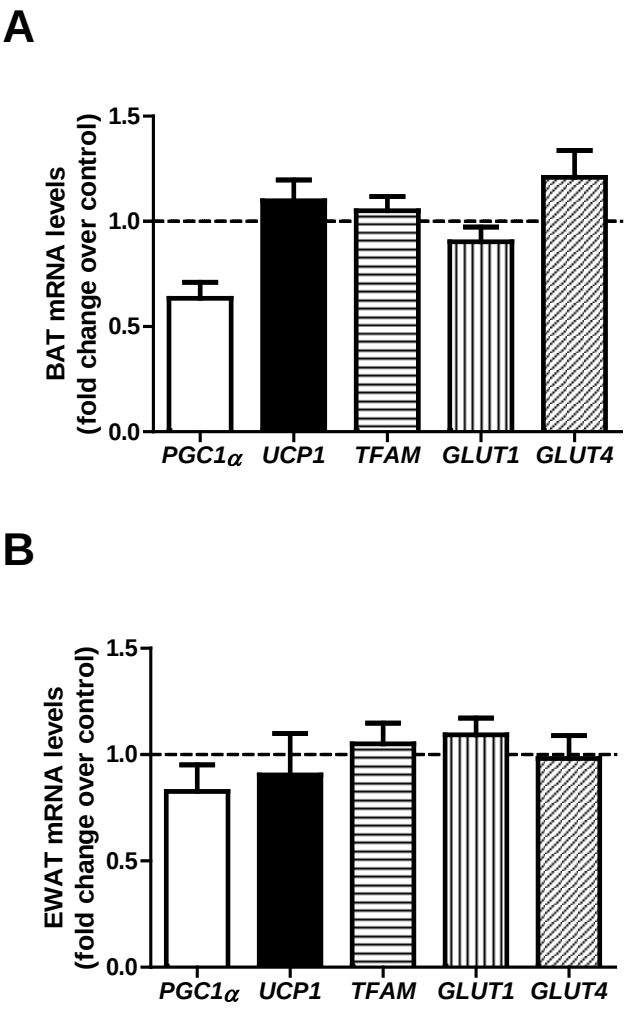
Supplementary Figure S6. Chronic BNP treatment does not change lipid levels and gene expression in liver of HFD-fed mice

(**A**) Total ceramides, (**B**) total diacylglycerols levels, and (**C**) mRNA levels of genes involved in fat oxidation and glucose metabolism in liver of saline- and BNP-treated HFD-fed mice.



Supplementary Figure S7. Expression of thermogenic and brown/beige gene markers in adipose tissues of db/db mice

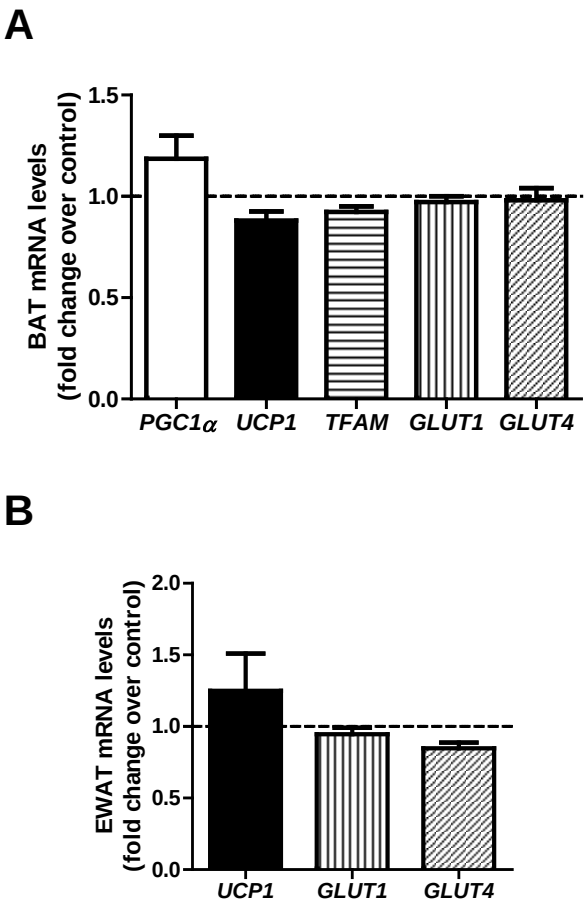
PGC1α, UCP1, TFAM, GLUT1 and *GLUT4* mRNA levels in (**A**) BAT and (**B**) EWAT of db/db mice treated for 4 weeks with BNP (n=8-10).



SUPPLEMENTARY DATA

Supplementary Figure S8. Expression of thermogenic and brown/beige gene markers in adipose tissues of HFD-fed mice

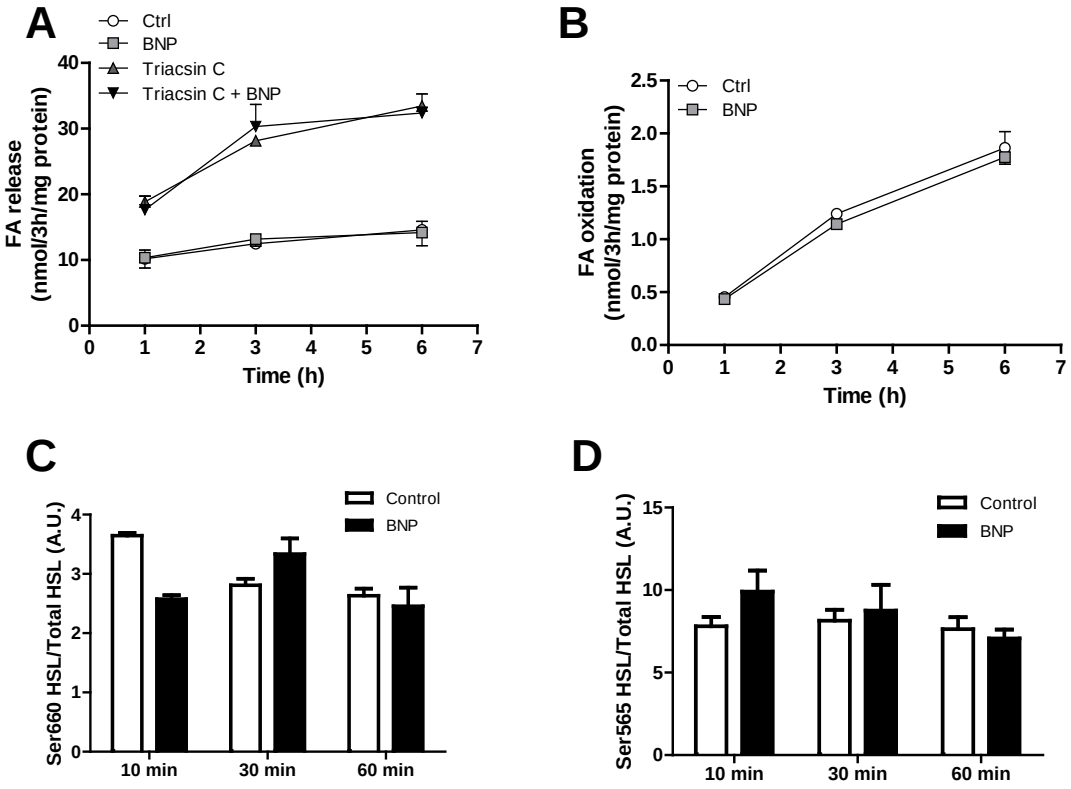
(**A**) *PGC1a, UCP1, TFAM, GLUT1* and *GLUT4* mRNA levels in BAT and (**B**) *UCP1, GLUT1* and *GLUT4* gene expression in EWAT of HFD mice treated for 4 weeks with BNP (n=8-10).



SUPPLEMENTARY DATA

Supplementary Figure S9. Effect of acute BNP treatment on lipolysis in human primary myotubes

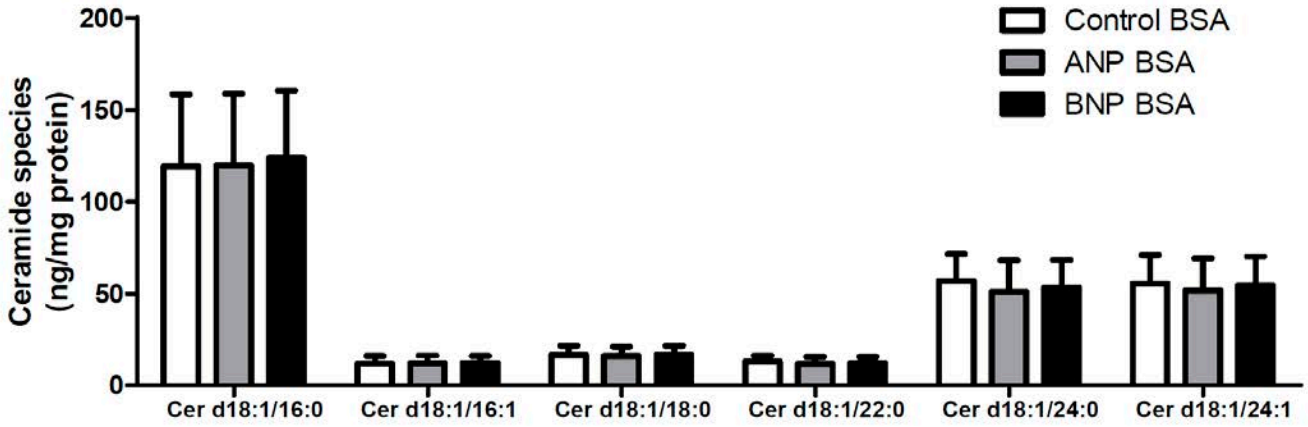
Time-course of (**A**) fatty acid (FA) release and (**B**) FA oxidation from endogenous pre-labeled TAG pools in response to 1, 3 or 6 hours BNP treatment in the presence or absence of triacsin C to block FA recycling into TAG pools. (**C**) HSL Ser660 and (**D**) HSL Ser565 phosphorylation were measured after 10, 30 and 60 min acute stimulation with 100 nM of BNP in human primary myotubes (n=3-5).



SUPPLEMENTARY DATA

Supplementary Figure S10. Effect of chronic NP treatment on basal ceramides content in human primary myotubes.

Ceramide species content in human primary myotubes in basal condition (BSA) in control myotubes and in response to 3-days treatment with 100 nM of ANP or BNP (n=4).



Author: Claire Laurens
Title: **From lipid droplet to intramuscular adipocytes: towards a causal link with insulin resistance ?**
Supervisor(s): Dr Cédric Moro, Dr Virginie Bourlier
Place and date of defense: Toulouse, 23 September 2016

My PhD research work was focused on the role of muscle lipids in the regulation of energy metabolism and insulin sensitivity. Lipids can be found under two different forms in skeletal muscle: adipocytes located between muscle fibers/bundles and lipid droplets inside muscle fibers (*i.e.* intramyocellular triacylglycerols or IMTG). These depots, when present in excess, have both been associated with insulin-resistance in humans, mainly because of intracellular lipotoxic lipid accumulation known to impair insulin signaling for IMTG, and through a yet unknown mechanism for adipocytes.

First, we isolated and characterized two distinct populations of progenitor cells from human muscle biopsies. The first population is composed of satellite cells (muscle progenitor cells) and display a myogenic differentiation potential *in vitro*. The second population is composed of cells that acquire the phenotypic and metabolic properties of functional white adipocytes, called fibro/adipogenic progenitors (FAPs). By using these cell models, we showed that FAPs-derived adipocytes secretions are able to impair insulin signaling and action in human skeletal muscle fibers *in vitro*. This paracrine effect could explain, at least partly, the inverse relationship observed between intramuscular adipocyte content and insulin sensitivity in humans.

Secondly, we studied the role of two proteins, *G_0/G_1 Switch Gene 2* (G0S2) and perilipin 5 (PLIN5), in lipid droplets dynamics as well as their impact on lipid metabolism and insulin sensitivity. We showed *in vitro* that these two proteins play a key role in the control of muscle lipolysis (*i.e.* IMTG hydrolysis) via the *adipose triglyceride lipase* (ATGL, catalyzing the limiting step of muscle lipolysis), and that G0S2 and PLIN5 inhibit ATGL activity through direct and indirect mechanisms, respectively. Furthermore, our data showed that G0S2 and PLIN5 invalidation *in vivo* in mouse skeletal muscle activates lipolysis, increases lipotoxicity and impairs insulin sensitivity. We have also highlighted an important role for PLIN5 in the regulation of fatty acids oxidation, by finely adjusting their availability to energy demand.

Overall, these results clearly show on one hand that a crosstalk between adipocytes and fibers within skeletal muscle can lead to an alteration of insulin sensitivity in humans, and on the other hand that G0S2 and PLIN5, two lipid droplet proteins, play a central role in the control of muscle lipid homeostasis and insulin sensitivity. These data help to develop our current understanding of the link between muscle lipids and insulin sensitivity in humans.

Keywords : metabolism • type 2 diabetes • insulin-resistance • skeletal muscle • lipid droplets • ATGL • lipolysis • G0S2 • PLIN5 • intramuscular adipocytes

Auteur : Claire Laurens

Titre : **De la gouttelette lipidique aux adipocytes intramusculaires : vers un lien causal avec l'insulino-résistance ?**

Directeur(s) de thèse : Dr Cédric Moro, Dr Virginie Bourlier

Lieu et date de soutenance : Toulouse, le 23 septembre 2016

Mon travail de thèse a été axé sur l'étude du rôle des lipides musculaires dans la régulation du métabolisme énergétique et la sensibilité à l'insuline. Les lipides sont présents sous deux formes au sein du muscle squelettique : soit sous forme d'adipocytes insérés entre les fibres/faisceaux musculaires, soit sous forme de gouttelettes lipidiques à l'intérieur des fibres musculaires (*i.e.* triglycérides intramyocellulaires ou IMTG). Ces deux dépôts de lipides, lorsqu'ils sont présents en excès, sont associés à la mise en place de l'insulino-résistance musculaire chez l'homme, via l'accumulation intracellulaire d'espèces lipidiques lipotoxiques altérant la signalisation insulinique pour les IMTG, et par un mécanisme inconnu pour les adipocytes.

Dans un premier temps, nous avons isolé et mieux caractérisé, à partir de biopsies musculaires humaines, deux populations de cellules progénitrices. La première population présente un potentiel de différenciation myogénique en culture, il s'agit des cellules satellites (cellules progénitrices musculaires). La deuxième population est composée de cellules capables d'acquérir les propriétés phénotypiques et métaboliques d'adipocytes blancs matures, il s'agit des progéniteurs fibro/adipocytaires (FAPs). Grace à ces modèles d'étude, nous avons mis en évidence que les sécrétions des adipocytes dérivés des FAPs sont capables d'altérer la voie de signalisation et les effets de l'insuline sur des fibres musculaires humaines *in vitro.* Cet effet paracrine pourrait en partie expliquer la corrélation négative observée entre le contenu en adipocytes intramusculaires et la sensibilité à l'insuline chez l'homme.

Dans un second temps, nous avons étudié le rôle de deux protéines, *G_0/G_1 Switch Gene 2* (G0S2) et la périlipine 5 (PLIN5), dans la dynamique des gouttelettes lipidiques ainsi que leur impact sur le métabolisme des lipides et la sensibilité à l'insuline. Nous avons montré *in vitro* que ces deux protéines jouent un rôle clé dans le contrôle de la lipolyse musculaire (*i.e.* hydrolyse des IMTG) via l'*adipose triglyceride lipase* (ATGL, enzyme limitante de la lipolyse musculaire), et que G0S2 et PLIN5 inhibent l'activité de l'ATGL par des mécanismes directs et indirects, respectivement. Par ailleurs, nos données ont montré que l'invalidation de G0S2 et PLIN5 dans le muscle squelettique active la lipolyse, augmente la lipotoxicité et diminue la sensibilité à l'insuline *in vivo* chez la souris. Nous avons également démontré un rôle important de PLIN5 dans la régulation de l'oxydation des acides gras en ajustant finement leur disponibilité aux besoins énergétiques des cellules.

En résumé, ces travaux démontrent d'une part qu'une communication entre adipocytes et fibres au sein du muscle peut entraîner une altération de la sensibilité à l'insuline musculaire chez l'homme, et d'autre part que G0S2 et PLIN5, deux protéines de la gouttelette lipidique, sont au centre du contrôle de l'homéostasie lipidique et du maintien de l'insulino-sensibilité musculaire. Ces données permettent ainsi d'élargir les connaissances existantes sur le lien entre les lipides musculaires et la sensibilité à l'insuline chez l'homme.

Mots-clés : métabolisme • diabète de type 2 • insulino-résistance • muscle squelettique • gouttelettes lipidiques • ATGL • lipolyse • G0S2 • PLIN5 • adipocytes intramusculaires